EFFECT OF IRON AND CAROTENOID CONCENTRATION ON IRON BIOAVAILABILITY IN PEA *Pisum sativum* L.

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ABSTRACT

Field pea (*Pisum sativum* L.) is a nutritious pulse crop consumed as food and feed all over the world. The present study was carried out to determine the potential effects of iron, phytate, and carotenoid concentrations on iron bioavailability of field pea seeds. PR-07 (recombinant inbred line (RIL) population derived from the cross Carrera/CDC Striker) segregated for iron concentration, and QTL were detected on LG3, LG4 and LG7, which combined explained 51 % of the phenotypic variance. In PR-07, iron concentration was positively correlated with iron bioavailability. In 4802-8 (derived from the cross 1-2347-144/ CDC Raezer) and 4803-4 (derived from the cross 1-150-81/ CDC Limerick) sub-lines, phytate concentration was negatively correlated with iron bioavailability. Four carotenoid compounds (lutein, violaxanthin, zeaxanthin and β-carotene) were measured in seeds of 4802-8 and 4803-4 sub-lines and summed to determine total carotenoid concentration. Green cotyledon and yellow cotyledon pea sub-lines did not differ significantly in total carotenoid concentration, but β-carotene concentration was greater in green cotyledon sub-lines. Although no significant correlation was detected between total carotenoid concentration and iron bioavailability, in 4802-8 sub-lines lutein concentration was positively correlated with iron bioavailability.

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LIST OF ABBREVIATIONS

ANOVA: analysis of variance

CDC: Crop Development Centre

cM: centiMorgan

CIM: composite interval mapping

DAF: days after flowering

DTF: days to maturity

DF: degree of freedom

DNA: deoxyribose nucleic acid

FEBIO: iron bioavailability

GBS: genotyping by sequencing

g: gram

IP_6: phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate)

Kg: kilogram

LG: linkage group

LOD: logarithm of the odds ratio

lpa: low-phytic acid

mg: milligram

min: minute

ml: milliliter

ns: not significant

ng: nanogram

nm: nanometer

PAM: pea association mapping panel

PA-P: phytate-phosphorus

P_i: inorganic phosphorus concentration

QTL: quantitative trait loci

RIL: recombinant inbred line

rpm: revolutions per minute

SD: standard deviation

SNPs: single nucleotide polymorphisms

SSR: simple sequence repeats

µg: microgram

µl: microliter

CHAPTER 1

INTRODUCTION

1.1 Background

Pulse crops are cool season, annually grown leguminous crops, which are harvested primarily for their mature seeds. Pulse crops are rich in dietary protein, slowly digestible starch, dietary fiber, essential vitamins, and minerals (Roy et al., 2010, Nwokolo., 1996). Major pulse crops grown in the world are common bean, dry pea, chickpea, and lentil.

Field pea (also known as dry pea) is among the world's oldest crops, having been grown in the Middle East from approximately 9000 years ago. In Canada, field pea has been cultivated over the past 100 years and many varieties have been developed for production (Goodwin, 2008). Canada is the largest global producer and exporter of field pea, with an average annual production of 3.2 million tonnes from 2010 and 2014 (Saskatchewan Ministry of Agriculture., 2013). Saskatchewan (67 %) is the largest producer of field pea among the Canadian provinces, followed by Alberta and Manitoba (Saskatchewan Ministry of Agriculture, 2013). Although pulse crop seeds are nutritious whole foods, they also contain varying concentration of antinutrients including polyphenolics, lectins, phytates, and protease inhibitors (Roy et al., 2010).

Iron is an essential micronutrient and its deficiency is a global nutritional problem. The daily requirement of iron for children, adult males, and adult females is 10 mg, 12 mg and 15 mg, respectively (Herbert, 1987). Antinutrients are compounds which block the absorption of nutrient minerals such as the iron in humans. Phytate or inositol hexakisphosphate (IP_6) is one of the antinutrients which is a strong chelator of divalent minerals, such as calcium, magnesium, zinc, and iron. Legumes account for 7.6 % of the annual global production of crop seeds/grains/fruits and 13.0 % of total phytate (Reddy et al., 1982). The amount of phytate varies from 0.4 % to 2.1 % in legume seeds (Reddy et al., 1982). The ability of phytate to chelate these

minerals may lead to reduced mineral bioavailability in human nutrition. Non-ruminant animals lack phytase enzymes in their digestive juices, so phytates are excreted along with bound minerals which cause environmental pollution. Low phytate crops could be beneficial for increasing the bioavailability of micronutrients and for reducing environmental pollution (Paik et al., 2003). Breeding of crops to enhance their nutritional value is known as biofortification (HarvestPlus program of the CGIAR system). Biofortification of crops has potential benefits for human health, the environment, as well as from an economic point of view. To develop *lpa* crops, synthesis of phytate is genetically altered during seed development (Raboy, 2001). Most *lpa* mutants have normal total phosphorus concentration but lower concentration of phytate, which means that mutants do not affect the absorption and transport of phosphorus from the soil, but block the phytate synthesis. The *lpa* mutants have been developed in several crops including corn (*Zea mays* L.), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), and soybean (*Gycine max* L.) (Raboy et al., 2000 Larson et al., 1998; Larson et al., 2000; Wilcox et al., 2000). With 65 % reduction of phytate, *lpa* corn was found to enhance absorption of iron by 49 %, zinc by 76 % and calcium by 43 % (Mendoza et al., 1998; Adams et al., 2000; Krebs et al., 2002). Two *lpa* mutants have been developed in pea (Warkentin et al., 2012). Iron bioavailability in *lpa* pea mutants was 1.4-1.9 times higher than in normal phytate pea varieties (Liu et al., 2014).

Phytate or inositol hexakisphosphate (IP_6) has been observed to reduce cancer in the lower intestinal tract, as well as reducing tumor formation and initial events of carcinogenesis (Pretlow et al., 1992; Shamsuddin et al., 1992; Sakamoto et al., 1993). Considering these beneficial effects of phytate, it should not be completely eliminated from the seeds of cereals and legumes. To enhance iron bioavailability in humans, it is necessary to determine whether other compounds have a positive effect on iron absorption from cereal-legume based foods.

β-carotene could potentially overcome the inhibitory effect of phytate on iron absorption. β-carotene supplementation doubled *in vitro* iron uptake compared to no β-carotene addition in corn and wheat flours (García-Casal et al., 2000; Layrisse et al, 1997). Green cotyledon pea cultivars had 10 times more β-carotene than yellow cotyledon cultivars (Ashokkumar et al, 2014). The potential association of phytate, iron, and carotenoid concentrations with iron bioavailability was determined in this study. PR-07 RIL population, which contrasts in cotyledon colour and iron concentration, was evaluated. Sub-lines from two pea breeding lines (4802-8 and 4803-4), which contrast in phytate concentration and cotyledon colour were also evaluated. An *in vitro* technique based on Caco-2 cells derived from human intestine (Wienk et al., 1999) was used to study iron absorption.

CHAPTER 2

LITERATURE REVIEW

The human body depends upon 49 micronutrient minerals and vitamins to ensure ideal metabolic activities (Welch and Graham, 2004). Malnutrition is a serious health issue in many countries and contributes immensely to child deaths. Approximately one third of the developing world population suffers from micronutrient malnutrition (Food and Agriculture Organization, 2012). Iron is one of the three critical nutrients, in addition to zinc (Zn) and vitamin A, which is the most limiting in human diets (World Health Organization, 2002).

2.1 Iron

Iron is one of the essential micronutrients and it performs many vital functions in the body. Iron is the main constituent of hemoglobin and myoglobin present in blood and muscle cells, respectively. Hemoglobin in erythrocytes is composed of four subunits, each with one heme group and one globular protein chain. The structure of hemoglobin allows distribution of oxygen from the lungs to the various tissues. Myoglobin, an iron containing oxygen storage protein present in muscles, has similar structure as that of hemoglobin, but with one heme unit and one globular protein unit. Cytochromes are iron containing enzymes containing heme groups. Cytochromes transport electrons in cells and cell organelles without permitting the reversible loading and unloading of oxygen. They transfer their energy in oxidative metabolism which takes place in mitochondria. Cytochromes also play an important role in the synthesis of steroids, bile acids, and signal control of some neurotransmitters. Iron is accumulated in the liver reversibly as ferritin and hemosiderin. It is transported within different compartments in the body by protein transferrin. The immune system as well as physical and mental growth requires an adequate amount of iron (FAO, 2001).

2.2 Iron deficiency

Iron deficiency is a major health concern and is highly prevalent in infants, menstruating and pregnant women, and adolescents of both sexes worldwide (FAO, 2001). In the weaning period, iron nutrition has great importance as it is required for development of the brain and for muscle development, which are differentiated in the early stages of life. Iron deficiency incidence in children age 1-2 of both sexes was 7 %, and for women age 16-19 was 19 % (Pollitt E., 1993). According to the World Health Organization, in developing countries, children younger than 5 years (39 %), between 5 and 14 years (48 %), all women (42 %), and all pregnant women (52 %) have anemia and half of them are affected by iron deficiency anemia. Iron deficiency anemia results in lower work productivity, increased child mortality, increased risk of maternal mortality, and susceptibility for many infectious diseases (Umbreit, 2005).

2.3 Causes of iron deficiency

Iron deficiency occurs when the physiological requirement of iron is not achieved by the bioavailable iron in the diet. It is most prevalent in populations dependent on plant-based diets as these have less bioavailable iron than diets that include meat. Cereal and legume-based diets contain low amounts of bioavailable iron, which may increase the risk of iron deficiency. About 30-40 % of iron in pork, liver, fish and 40-60 % of iron in beef, lamb, and chicken is heme iron, 15-35 % of which can be absorbed (Monsen et al, 1978). On the other hand, plant foods contain non-heme iron and its absorption is less than 10 %. The absorption of non-heme iron can be enhanced by meat or ascorbic acid and can be reduced by phytate, polyphenols or calcium (Zimmermann et al, 2007).

Iron deficiency is even more prevalent in adult females due to menstrual blood loss every month. A 1 ml loss of blood translates to a loss of 0.5 mg of iron. There is heavy blood loss (80 ml per month) in 10 % of women. Iron requirement is three-fold greater during pregnancy.

Without supplements, many women are unable to store the required amount of iron. In infants, there is risk to iron deficiency as their rapid growth exhausts the iron stored during gestation (250 mg), if fortified food products are not consumed (Zimmermann et al, 2007).

Parasite infection with *Trichuris trichiura* (whipworm) and *Necator americanus* (hookworm) may also cause iron deficiency. Daily blood loss in children affected by these parasites is 0.005 ml/worm. The infestation of thousands of parasites is common so can contribute to iron deficiency anemia (Layrisse et al., 1967). More than 700 million people in tropical and subtropical regions are affected by hookworms (Zimmermann et al, 2007).

2.4 Strategies to alleviate iron deficiency

Supplementation. Supplementation consists of the use of pills, capsules, and syrups in order to provide the optimal dose of specific nutrients in a highly absorbable form, and is generally a cost effective means to address a deficiency. Ferrous sulphate and ferrous gluconate are the ferrous iron salts recommended for oral supplementation. A ferrous sulphate tablet (300 mg containing 60 mg of iron taken 3 to 4 times a day) is prescribed as standard treatment. It has attributed to increased birth weight and reduced preterm delivery, but was not effective in moderating the incidence of anemia during the third trimester of pregnancy (Zimmermann et al, 2007). Supplemental vitamin A may degrade during baking, so the timing of supplementation played an important role. Vitamin A supplementation before baking bread had no enhancing effect on iron absorption, whereas supplementation immediately before consumption had a significant enhancing effect on iron absorption (García-Casal et al., 1998). In a recent study, different molar ratios of vitamin A to ferrous fumarate/NaFe-EDTA (iron) (10:1, 20:1 and 40:1) were used to evaluate iron uptake by Caco-2 cells; iron absorption was not significantly affected by these different molar ratios (García-Casal and Leets, 2014).

Fortification. Fortification is a strategy to improve the nutritional quality of food products by adding specific micronutrients (Haas and Miller, 2006). It is also a sustainable and cost effective method to combat iron deficiency through the fortification of staple cereal flours with high bioavailability iron compounds such as ferrous sulphate. However, ferrous sulphate is soluble in water and strongly interacts with food components to give off-flavors, color changes, or fat oxidation (Zimmermann et al, 2007). This makes it necessary that iron-fortified cereal flours are consumed shortly after milling. This is of concern in developing countries where cereal flour is often stored for long periods. Thus, the iron fortification of flours is performed with less soluble forms such as elemental iron powders which are only half as well absorbed compared to ferrous sulphate. A decline in the prevalence of anemia and iron deficiency was observed in school age children in south India with dual fortification of salt with iodine and iron (Andersson et al, 2008)

Biofortification. Biofortification is a practice to enhance the nutritional value of staple food crops through plant breeding. Biofortification primarily targets low income households, but is also attractive to persons with higher income who want highly nutritious plant-based foods. The combination of plant breeding with new approaches of biotechnology has resulted in the development of staple crops enriched with nutrients (Nestel et al, 2006). Biofortified maize, rice, and barley have been produced with increased concentration of iron, zinc, or provitamin A (Raboy et al., 2000; Larson et al., 2000; Larson et al., 1998).

2.5 Antinutrients

Plant foods contain many antinutrients, which reduce the absorption of micronutrients during digestion (Welch and Graham, 1999). The nutritional quality of food crops can be enhanced by lowering the concentration of antinutrients. Plants produce chemical compounds as part of their defense strategy for herbivores, microorganisms and viruses. These compounds can

either be toxic (i.e. lectins, glycosides, alkaloids), unpalatable or indigestible (i.e. tannins, saponins), or antinutritive (i.e. phytates) (Enneking and Wink, 2000).

Phytate is the primary storage form of phosphorus in seeds. As a macronutrient, phosphorus is required for plant growth and metabolism. Phytate is utilized during germination by the chemical action of endogenous phytase and other phosphatases. It constitutes about 1.5 % of seed dry weight in cereal and grain crops (Bohn et al, 2008). Phytate is a mixed cation salt of phytate and known as myo-inositol-1, 2, 3, 4, 5, 6 hexakisphosphate (IP_6) (Cosgrove, 1980). It constitutes about 60-80 % of the total amount of phosphorus in seeds. The majority of phosphorus absorbed from the soil is converted to phytate/IP_6 which is the major pool in the flux of phosphorus through the agricultural ecology (Raboy et al, 2001). Annually, about 65 % of the phosphorus fertilizers applied to crops is converted into phytate (Lott et al, 2001).

2.6 Polyphenols

Polyphenols reduced iron bioavailability by forming non-absorbable complexes in common bean (Petry, 2010). Traditional methods such as soaking (18 hours) and germination (48 hours) reduced the concentration of polyphenols in pea seeds by 52 % and 88 %, respectively. Soaking with dehulling, and pressure cooking leached out 76 % of the polyphenols. (Bishnoi et al., 1994). In a study evaluating the effect of phenolic compounds in common beans, some polyphenols (catechin, 3, 4-dihydroxybenzoic acid, kaempferol, and kaempferol 3-glucoside) had an enhancing effect on iron bioavailability, whereas others (myricetin, myricetin 3-glucoside, quercetin, and quercetin 3-glucoside) showed inhibitory effects (Hart et al., 2015).

2.7 Phytate/ IP_6 as an antinutrient

Ins P6 possesses negatively charged sites, and thus acts as a strong chelator of metallic cations like potassium, iron, calcium, zinc, magnesium, and manganese. Consequently, a mixed salt is formed, which is largely excreted by humans and other non-ruminant animals which lack

phytase enzymes for phytate hydrolysis. Due to the unavailability of the bound minerals, mineral deficiencies arise which are also known as “hidden hunger”. Mainly developing countries suffer because of their dependence on cereal and legumes as their staple foods (Warkentin et al, 2012). Antinutrients result in two major problems: the reduction of nutritional value of the food leads to mineral nutrient deficiency problems including anemia and osteoporosis; and the excreted animal waste may cause eutrophication in water ways.

2.8 Reducing phytate concentration using phytase

To reduce phytate content in foods, phytase enzyme is produced from transformed wheat (phytase gene from *Aspergillus niger*) (Brinch-Pedersen et al., 2000), and microorganisms (transgenic strain of *Bacillus mucilagenosus*) (Li et al, 2007) to use as food additives (Vohra and Satyanarayana, 2003). Significant results have been obtained with supplementation of microbial phytase to pig diets, which has enhanced phosphorus availability from 18-56 % in maize, from 62-74 % in wheat, and from 52-67 % in triticale (Düngelhoef et al, 1994). Phytate degradation in cereal porridges increased the bioavailability of iron (Hurrell et al, 2002).

Traditional methods including fermentation, sprouting/germination, and soaking are also used to reduce phytate content in cereals and legumes by activating endogenous phytase (Sandberg and Svanberg, 1991). Sprouting reduced phytate concentration in pigeon pea by 35 % to 39 % (Duhan et al, 2002). Sprouting also increased the activity of phytase and degraded phytate in rye (79 %) barley (80 %), and rice (71 %) (Larsson and Sandberg, 1992).

2.9 Reducing phytate concentration through plant breeding

Plant breeding addressed the challenge of feeding the world’s population through the “Green Revolution”. But the challenge was focused on high yielding crop varieties and less concerned about their nutritional value. Consequently, this revolution reduced the extent of the starving population, but on the other hand, resulted in widespread micronutrient malnutrition

(Welch and Graham, 1999). Agriculture must concentrate not only on producing high quantity but also high quality nutritious foods (Bouis and Welch, 2009). Biofortification is being implemented to address micronutrient malnutrition (hidden hunger) worldwide. HarvestPlus, as a program of the Consultative Group for International Agricultural Research (CGIAR), was officially launched in 2004, and has emerged as a global leader in developing biofortified crops. Its prime objective is to reduce hidden hunger directly through staple foods including cereals and legumes.

2.10 Low phytate crop varieties

Alteration in the biochemical pathway of phytate has been performed through mutagenesis followed by conventional breeding. Low phytate mutants for crops including maize (*Zea mays* L.), soybean (*Glycine max* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), common bean (*Phaseolus vulgaris* L.) and barley (*Hordeum vulgare* L.) have been developed. Two types of mutants have been found in maize, i.e., *lpa*1-1 mutants, which have reduced phytic acid phosphorus concentration (PA-P) and increased inorganic phosphorus (P_i) concentration, and *lpa*2-1 mutants, which additionally have at least three other forms of myoinositol phosphates. In corn, two *lpa* mutants (*lpa*1-1 and *lpa*2-1) showed reduction in PA-P by 50-66 % accompanied by an increase in P_i (Raboy et al, 2000). Likewise, in barley, two low phytate mutants i.e. *lpa*1-1 and *lpa*2-1 were developed with 48 % and 41 % reduction in total soluble inositol phosphate, respectively (Rasmussen et al, 1998, Dorsch et al, 2003). The *lpa* mutants obtained for soybean (Wilcox et al, 2000) and rice (Liu et al, 2007) had up to 80 % and 64 % reduction in PA-P, respectively. Wheat with a 43 % reduction in PA-P, had increased bioavailability of Fe and Zn, and accumulation of inorganic phosphorus increased in the endosperm and diminished in the bran (Guttieri et al, 2004). White seed coat common bean lines with reduced tannin and

polyphenol concentration, in addition to the *lpa* trait had twelve-fold increase in iron bioavailability, as observed using the Caco-2 cell model (Campion et al, 2013).

Two low phytate mutants (1-150-81 and 1-2347-144) of field pea cultivar CDC Bronco were developed through chemical mutagenesis at the Crop Development Centre, University of Saskatchewan (Warkentin et al, 2012). Genotype by environment interaction was studied for the two low phytate pea lines (1-150-81 and 1-2347-144) along with their progenitor (CDC Bronco) and two check varieties (Cutlass and CDC Golden) at three diverse field locations in Saskatchewan (Delgerjav, 2012; Warkentin et al, 2012). Emergence count, plant height, mycosphaerella blight score and lodging score did not differ between the *lpa* lines and check cultivars. Genotype by environment interaction was significant for flowering, days to maturity, grain yield, seed weight, concentration of phytate phosphorus, inorganic phosphorus, and concentration of iron. The two low-phytate lines were moderately delayed in flowering (2-4 days) and maturity (1-4 days) compared to the check cultivars. Seed weight of the low phytate lines was significantly lower than CDC Bronco. The two low phytate lines 1-2347-144 and 1-150-81 yielded 92 % and 86 % of CDC Bronco, respectively. The concentration of phytate phosphorus was considerably reduced in 1-2347-144 (1.13 mg g^{-1}) and 1-150-81 (1.20 mg g^{-1}) in comparison to the other cultivars, which ranged from 2.94-2.99 mg g^{-1}. As a result of low phytate phosphorus concentration, there was a commensurate increase in inorganic phosphorus. Lines 1-2347-144 (1.22 mg g^{-1}) and 1-150-81 (1.28 mg g^{-1}) had higher inorganic phosphorus concentration than the other three cultivars which ranged from 0.24-0.26 mg g^{-1}. Iron concentration in low phytate pea lines was 42.1 mg kg^{-1} as compared to 39.4 mg kg^{-1} in CDC Bronco. Environment influenced iron concentration ranging from 35.1 mg kg^{-1} (Outlook 2010) to 57.0 mg kg^{-1} (Saskatoon 2010) (average of all lines). The two *lpa* mutant lines and CDC Bronco

showed no significant difference for percent emergence, days to flowering, plant height, mycosphaerella blight score, lodging and days to maturity for two year (2010 and 2011) field trial at Saskatoon and Rosthern (Shunmugam, 2012).

The *lpa* lines 1-150-81 and 1-2347-144 were studied for inheritance of the low phytate trait through crosses between these lines with two normal phytate pea lines. F_1 progeny had normal phytate concentration, while the F_2 populations fit into the expected phenotypic ratios (i.e. 3 normal: 1 low phytate) for single recessive gene control (Rehman et al, 2012).

A recombinant inbred lines (RIL) population PR-15, consisting of 163 lines was developed at University of Saskatchewan by crossing cultivar CDC Meadow (normal phytate) and 1-2347-144 (low phytate line) followed by single seed decent under greenhouse conditions (Shunmugam et al. 2015). In 2012 and 2013, PR-15 was phenotyped in two replicate experiments at two locations in Saskatchewan (Saskatoon and Rosthern). DNA was extracted from leaf tissues of PR-15 and genotyped using a 1536 SNP marker Illumina GoldenGate assay developed at National Research Council of Canada and University of Saskatchewan (Sindhu et al. 2014). A QTL (Quantitative Trait Locus) associated with phytate phosphorus concentration was identified in PR-15 (Shunmugam et al. 2015).

The two low phytate lines (1-150-81 and 1-2347-144) along with their progenitor cultivar CDC Bronco and two check varieties (CDC Meadow and CDC Golden) were analyzed for iron bioavailability (Liu et al. 2014). All the varieties had similar concentration of iron, but the low phytate pea lines had higher bioavailable iron and inorganic phosphorus as compared to normal phytate cultivars. A QTL (Quantitative Trait Locus) associated with iron bioavailability was identified and found to be located at same locus as phytate concentration on LG5 (Shunmugam et al. 2015). Pigmented seed coat varieties with high iron concentration were found to have 7

times lower iron bioavailability than non-pigmented seed coat varieties of pea. Pigmented rice genotype, Tong Lan Mo Mi had the highest Fe concentration of all genotypes tested, but showed the lowest bioavailable Fe concentration, showing that some polyphenolic compounds have an effect on bioavailability of iron (Glahn et al. 2002). Although the molar ratio of PA: Fe can predict the bioavailable amount of iron, when PA: Fe exceeds 10:1, the phytate concentration had maximal inhibition effect on iron bioavailability (Glahn et al., 2002a).

2.11 *In vitro* digestion/Caco-2 cell culture assay for iron bioavailability:

The human colon adenocarcinoma cell line Caco-2 showed structural and functional differentiation similar to mature enterocytes (Pinto et al., 1983). It is a promising tool to study uptake and transport of bile salts, vitamins, amino acids and drugs. It differentiates spontaneously and is suitable as an *in vitro* model for absorption studies (Misfeldt., 1976; García-Casal et al., 1996). In the Caco-2 cell culture assay, simulated peptic digested food is fed to Caco-2 cell monolayers (Figure 1 in Glahn et al.,1998).

A fair assessment of *in vivo* absorption of various compounds can be accomplished by measuring permeability across Caco-2 cell monolayers (Yee S., 1997). Iron uptake by Caco-2 cells depends on iron form (ferrous or ferric) and substances which inhibit (such as phytates and tannins) or enhance (such as ascorbic acid) iron absorption (Yun et al., 2004; Cook and Reddy, 2001). Iron bioavailability is determined by measuring ferritin accumulated by Caco-2 cells monolayers (García-Casal et al., 1996, Glahn et al., 1998). Due to the human origin of the Caco-2 cells, many human nutrition studies were significantly correlated with Caco-2 cells results. García-Casal et al (1996) observed that human studies and Caco-2 cells showed similar iron uptake in the presence of various proteins (soybean protein, egg albumen and bovine serum albumen). During iron uptake experiments, the days of Caco-2 cells in culture and their

contamination were important issues. Sixteen days of cells in culture showed higher iron uptake than 8 days in culture (García-Casal et al., 2000).

2.12 Carotenoids in pea

Plants synthesize natural pigments including carotenoids, which are beneficial for humans. β-carotene, the precursor for vitamin A, is an important carotenoid in plants. β-carotene is converted to retinal, which is the fundamental structure for transduction of light into visual signals, thus has an important role in vision. Vitamin A acts as an anti-inflammatory agent as well as an immune modulator and thus enhances the immune system (Reifen., 2002, Semba., 1998). Studies for iron absorption were conducted using carotenoids without provitamin-A activity (such as lycopene, lutein and zeaxanthin) in wheat and corn based breakfast. Iron absorption had significantly increased by 2-3 times with supplementation of these carotenoids (Table 3 in García-Casal., 2006). Cereal fortification with carotenoids with provitamin-A activity such as β-carotene and with vitamin A also lead to higher iron absorption (García-Casal et al, 1998).

Iron solubility increased in the presence of vitamin A at pH 6 (García-Casal et al, 1998). Vitamin A and β-carotene each form a soluble complex with iron, which prevents iron from binding with phytate and polyphenols (García-Casal et al, 1998). In Venezuelan fortification program, vitamin A doubled iron absorption by counteracting the inhibitory effect of phytate in corn-based meals (Layrisse et al, 1997); however, this finding has not been corroborated by further studies in healthy subjects (Walczyk et al., 2003).

During a Venezuelan wheat, rice and corn fortification program, β-carotene (6 µmol/L) supplementation increased iron uptake (114.9± 6.3 pmol/mg cell protein) compared to no β-carotene addition (47.2± 5.9 pmol/mg cell protein) in Caco-2 cells with pH 5.5 at 16 days in culture. β-carotene (0.9 µmol/ L) had been observed to overcome the inhibitory effect of tannins

(2.1 µmol/L and 4.2 µmol/L) and phytate (García-Casal et al, 2000). Synergistic interactions in absorption were also observed among iron, zinc and vitamin A (Graham and Rosser., 2000).

Carotenoid concentration was compared in 12 pea cultivars grown in field trials at 4 locations for two years (2009 and 2010) in Saskatchewan. The greatest concentration of carotenoids was found in cotyledons, which comprised about 90 % of the mass of the whole seed. Green cotyledon cultivars had 10 times more β-carotene than yellow cotyledon cultivars (Table 2 in Ashokkumar et al., 2014).

Green cotyledon cultivars (16-21 mg kg^{-1}) were richer in total carotenoids than yellow cotyledon cultivars (7-12 mg kg^{-1}). On average, the pea cultivars evaluated had highest concentration of lutein (11.45 mg kg^{-1}) followed by violaxanthin (0.52 mg kg^{-1}), β-carotene (0.47 mg kg^{-1}) and zeaxanthin (0.16 mg kg^{-1}) (Ashokkumar et al, 2014). Location had a significant effect on average total carotenoid concentration ranging from 11.90 mg kg^{-1} (Rosthern 2009) to 14.06 mg kg^{-1} (Sutherland 2010) (Ashokkumar et al, 2014).

Sixteen rice genotypes were compared for their iron bioavailability (Glahn et al., 2002). Interestingly, the genotypes which showed low bioavailability of iron, had kernel purple or brown color compared to the genotypes with higher bioavailability of iron which had white kernel color, i.e., the presence of polyphenols decreased the bioavailability of iron (Glahn et al, 2002). However, it has yet to be confirmed whether greater concentration of carotenoids has any profound effect on iron bioavailability in pulse crops.

2.13 QTL mapping for iron concentration in PR-07 RIL population

PR-07 is a population of 142 recombinant inbred lines (RILs) obtained from a cross between Carrera (yellow cotyledon, susceptible to mycosphaerella blight and lodging) and CDC Striker (green cotyledon, moderately resistant to mycosphaerella blight and lodging) (Warkentin et al., 2002, Liu, 2012). PR-07 was developed to characterize mycosphaerella blight resistance,

lodging resistance, and micronutrient concentration. PR-07 consists of 69 lines with yellow cotyledons, 67 lines with green cotyledons, and 6 lines which are mixed for yellow and green cotyledon colour. Iron concentration of harvested seeds of PR-07 lines was measured using Atomic Absorption Spectrometry (AAS) for two locations (Saskatoon and Rosthern, SK, Canada) with 2 replications per location for each of three years (2010, 2011 and 2012) (Liu, Y., 2012; Liu, unpublished). Mean iron concentration of parents Carrera and CDC Striker were 47 mg kg^{-1} and 42 mg kg^{-1}, respectively (Figure 2.1). Mean iron concentration of the RILs ranged from 40-57 mg kg^{-1} at Saskatoon and from 39-59 mg kg^{-1} at Rosthern (Figure 2.) (Liu, Y., 2012; Liu, unpublished). Coefficient of variation ranged from 9.3 to 13.8. RILs differed significantly for iron concentration (Table 2.1 and Figure 2.2) (Liu, 2012; Liu, unpublished). Lines were segregating and significantly (F (5.6, $P<0.001$) different from each other for iron concentration (Figure 2.2 and Table 2.1).

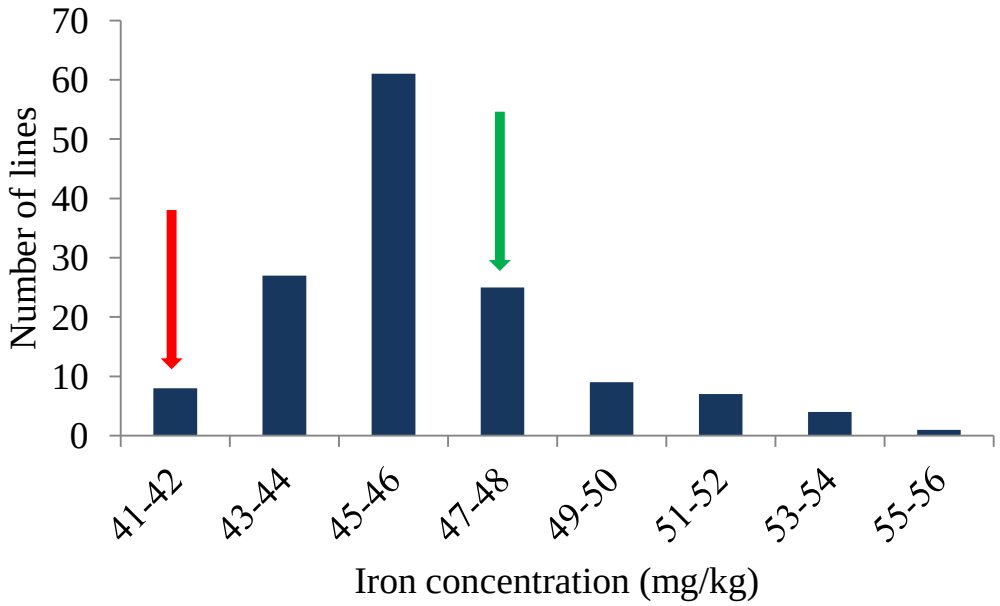

Figure 2.1 Frequency distribution of the average of least square means of iron concentration over three years (2010, 2011 and 2012) and two locations (Saskatoon and Rosthern) for the Recombinant Inbred Lines of Carrera X CDC Striker populations (adapted from Liu, 2012; Liu, unpublished) Red arrow: CDC Striker; Green arrow: Carrera

Table 2.1 Analysis of variance for iron concentration in pea recombinant inbred line population (PR-07) derived from a cross between Carrera and CDC Striker evaluated at Saskatoon and Rosthern in 2010, 2011 and 2012 (adapted from Liu, 2012; Liu, unpublished).

Effect	Num DF	F-value Iron Concentration
Genotype	143	5.65*
Year	2	162.28*
Location	1	17.78*
Genotype*Year	282	0.94^{ns}
Genotype*Location	141	1.26^{ns}
Genotype*Location*Year	284	3.89*

Notes: DF, degrees of freedom; ns, not significant; *, significant at $p<0.001$.

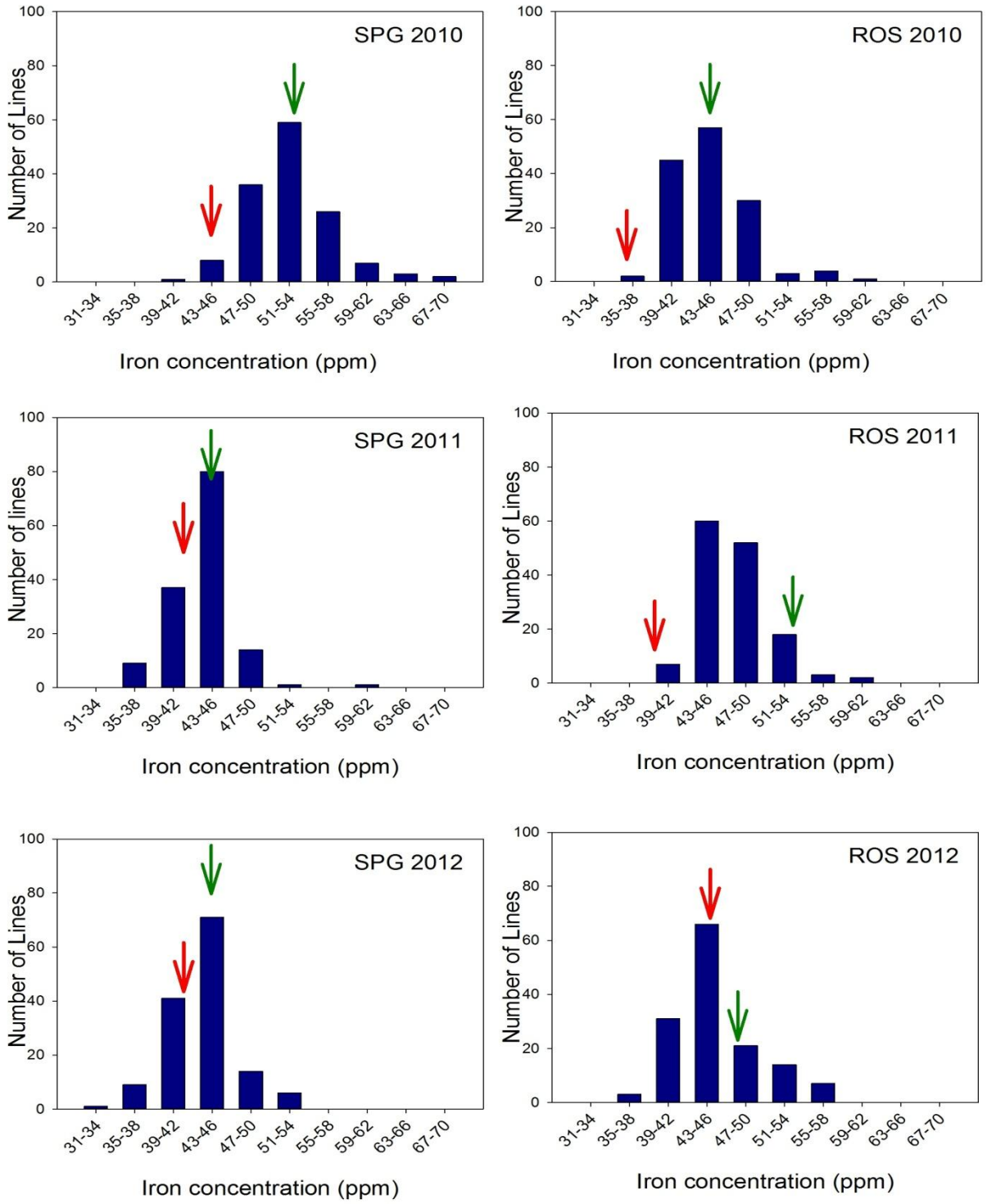

Figure 2.2 Frequency distribution of the least square means of iron concentration over three years (2010, 2011 and 2012) and two locations (Saskatoon and Rosthern) for the Recombinant Inbred Lines of Carrera X CDC Striker populations (adapted from Liu, 2012; Liu, unpublished) Red arrow: CDC Striker; Green arrow: Carrera

A genetic map with 288.3 cM coverage was generated with 56 SSR markers and QTLs associated with iron concentration in PR-07 were identified (Liu, 2012). The QTL on LGIII (Q3-1), between markers AA491 and AB44 (19.3 cM), was associated with several traits including iron concentration with phenotypic variation explained from 9.6 to 16.5 %. A QTL (Q4-2) on LGIV-1 was associated with iron concentration at Saskatoon in 2011 (Liu, 2012).

Five RIL populations, one of which was PR-07, were genotyped with a pea GoldenGate array (Ps1536 OPA) developed at the University of Saskatchewan (Sindhu et al., 2014). In PR-07, 388 SNP markers were polymorphic among parental lines and 245 were mapped (Sindhu et al., 2014). From these markers, linkage groups were determined by Carthagene 1.2.2 (De Keyser et al. 2010) at a LOD (logarithm of odds ratio) score of 3.0 with a maximum distance between two markers of 50 cM (Kosambi). Recently a GBS (Genotyping by sequencing) method was used to genotype and develop a linkage map for PR-07 population with 3131 SNP markers representing 891 loci in 12 linkage groups (Figure 3.1) (Gali, unpublished).

2.14 Project Hypotheses

1. QTL associated with iron concentration will be fine mapped using a dense linkage map of a pea recombinant inbred line population segregating for iron concentration.
2. Pea seeds with greater iron concentration will have greater iron bioavailability.
3. Pea seeds with greater carotenoid concentration will have greater iron bioavailability.
4. Low phytate concentration and high carotenoid concentration in pea seeds will have additive benefits for iron bioavailability.

2.15 Project Objectives

1. To map QTL associated with iron concentration and compare it between linkage maps obtained from GoldenGate and GBS technology.
2. To compare iron bioavailability in pea lines contrasting in iron concentration.
3. To compare iron bioavailability in pea lines contrasting in phytate concentration and cotyledon color.

CHAPTER 3

ANALYSIS OF QTL ASSOCIATED WITH IRON CONCENTRATION AND IRON BIOAVAILABILITY IN PR-07 LINES

3.1 Introduction

Based on iron concentration data from the Pea Association Mapping (PAM) panel developed at the University of Saskatchewan (Diapari et al., 2015), which contains cultivars CDC Striker (Warkentin et al., 2004) and Carrera, PR-07 RILs (derived from the cross Carrera/CDC Striker) were expected to segregate for iron concentration. Average data from three years (2010, 2011 and 2012) and two locations (Saskatoon and Rosthern) showed iron concentration in PR-07 RILs varied from 41 mg kg^{-1} to 58 mg kg^{-1} (Figure 2.1). Data were used to identify QTL associated with iron concentration (Liu, 2012; Liu, unpublished). QTL analysis of iron concentration in PR-07 was initially conducted based on Simple Sequence Repeats (SSR) markers (Liu, 2012). Later these RILs were genotyped using the Ps1536 Illumina GoldenGate array developed by Sindhu et al. (2014). Recently, PR-07 was further genotyped using Genotyping by Sequencing (GBS). This GBS data were integrated with the GoldenGate and SSR data to produce a linkage map consisting of 3131 markers (Kishore Gali et al, unpublished).

The next goal was to determine the effect of iron concentration on iron bioavailability. Iron bioavailability was measured in selected 40 RILs which contrast for iron concentration using the *in vitro* digestion/Caco-2 cell culture assay. The Caco-2 cell model mimics human gastric and intestinal digestion, with monolayers of human intestinal epithelial cells. The application of Caco-2 cell model includes screening of different non-radiolabeled food digests for iron bioavailability. In this model, the amount of ferritin produced by Caco-2 cells accurately predicts food iron availability (Glahn et al., 1998). Iron uptake is determined from ferritin formation in Caco-2 cells monolayers in response to exposed digest of field pea samples.

3.2 Hypotheses

1. QTL associated with iron concentration will be fine mapped using a dense linkage map of a pea recombinant inbred line population segregating for iron concentration.
2. Pea seeds with greater iron concentration will have greater iron bioavailability.

3.3 Objective

1. To map QTL associated with iron concentration and compare it between linkage maps obtained from GoldenGate and GBS technology.
2. To compare iron bioavailability in pea lines contrasting in iron concentration.

3.4 Material and methods

3.4.1 QTL analysis using GoldenGate array linkage map

Quantitative data for iron concentration from 136 PR-07 recombinant inbred lines (from Liu, 2012; Liu, unpublished) were used for QTL mapping. In 2014, genotypic data for these 136 lines were obtained from the PR-07 linkage map developed with GoldenGate array technology (Sindhu et al., 2014). This linkage map, the phenotypic data, and QTL Cartographer were used to determine the location of QTLs associated with iron concentration. Composite interval mapping (CIM) was performed using GoldenGate markers (Sindhu et al., 2014) and the heterozygous threshold value was set at 10 % with 1000 permutations at 0.05 significance level.

3.4.2 QTL analysis using GBS and Goldengate array linkage map

In 2016, a dense linkage map was developed through GBS technology. PR-07 RIL population was segregating for iron concentration and cotyledon color. Therefore, the PR-07 GBS linkage map was used for identification of QTL associated with iron concentration and cotyledon color. Mean (of 12 location-years, i.e., one data point) iron concentration of each PR-

07 line (Liu, 2012: Liu unpublished) was used in QTL Cartographer to identify QTL associated with iron concentration. Composite interval mapping (CIM) was performed using GBS and GoldenGate markers (Kishore Gali et al., unpublished) and the heterozygous threshold value was set at 10 % with 1000 permutations at 0.05 significance level.

3.4.3 Determination of the iron concentration of field pea samples

PR-07 RILs which showed the lowest and highest iron concentration (20 each) with least standard deviation were selected. These lines were selected based on their cotyledon color as well, as the parents (Carrera and CDC Striker) differ for this trait (Table 3.1).

Table 3.1 Selection of PR-07 RILs based on their cotyledon color (green and yellow) and iron concentration (low and high) (from Liu, 2012).

Cotyledon colour	Iron concentration	Mean ± SD	Number of lines selected
Green	Low	42.6 ± 0.92	10
Green	High	49.9 ± 2.18	10
Yellow	Low	42.9 ± 1.84	10
Yellow	High	51.0 ± 2.10	10

SD: standard deviation.

The iron concentration of these selected 40 PR-07 RILs was measured using atomic absorption spectroscopy (AAS) in the Department of Plant Sciences, University of Saskatchewan. The procedure of sample digestion was based on Thavarajah et al (2007). Dried peas were ground into fine powder (<0.5 mm sieve), then 200 mg of fine powder and 3 ml HNO_3 was poured into a digestion tube. For complete digestion of samples, tubes were heated to 70-80°C. Then, 0.5 ml of 30 % H_2O_2 was added in to the tubes and tubes were vortexed for 2 min. Millipore water was used to dilute sample solution to make volume 25 ml. The digested samples were assessed for iron concentration using AAS. These digested samples were assessed for iron concentration using AAS in collaboration with Mr. Barry Goetz in the Plant Sciences Department, University of Saskatchewan.

3.4.4 Determination of the phytate concentration

Wade's reagent method (Gao et al., 2007) was used to test the phytic acid phosphorus (PA-P) concentration in the selected PR-07 samples with four technical repeats. These tests were conducted in the Plant Sciences Department, University of Saskatchewan. The reagents used were: 0.8N H Cl: 10 % Na_2SO_4, 10 % NaCl and Wade's reagent (0.03 % $FeCl_3$ $6H_2O$: 0.3 % sulfosalicylic acid). A total of 50 mg of ground (Retsch Model ZM200, Newtown, PA, USA; 0.5 mm particle size, 1800 g) sample from each line as placed in a tube and 1 ml of 0.8N HCl: 10 % Na_2SO_4 was added. The aliquots were put on a shaker for 16 hours, and then centrifuged at 3000 g for 20 minutes. Thirty µl of extract was placed in a new tube and 720 µl of double distilled water and 250 µl of Wade's Reagent were added and the tube was vortexed for 10 seconds. A 200 µl aliquot was placed in microtitre plate wells, and the absorbance values were read at 540 nm using a microplate reader (Bio-Rad Benchmark, Hercules, CA, U.S.A.). A stock solution containing 1 mg phytate phosphorus per ml was prepared by dissolving 549.9 mg phytate dodecasodium salt hydrate (Sigma-Aldrich Co., St. Louis, MO, USA) in 100 ml water. This stock solution was used to prepare standard solutions with 25, 50, 100, 200, 300, 400, 500 and 600 ul phytate phosphorus per ml. The standard curve was used to obtain the value for phytate phosphorus per 30 µl of the 1 ml total extract. This value was converted to mg of phytate phosphorus (PA-P) g^{-1} as follows:

mg PA-P g^{-1} = (µg PA-P in assay) x (1 ml extract/0.03 ml assayed colorimetrically) x (1/50 mg tissue) x (1/1000)

3.4.5 Caco-2 cell culture assay

This technique was developed in the laboratory of Dr. Raymond Glahn at USDA-ARS, Cornell University, Ithaca, New York. Caco-2 studies on pea samples were conducted in Dr. Glahn's laboratory.

Twenty gram of field pea seeds was weighed in 250 ml conical flask. Seeds were rinsed thrice with 18 ohm deionised water. Sixty ml of deionised water was poured into samples in a conical flask and latter covered with aluminum foil. These conical flasks filled with sample and deionised water were arranged in plastic tub to place in an autoclave and cooked with "liquid cycle" for 30 min. After cooking, samples were poured into aluminum dishes and covered with aluminum foil. Aluminum dishes were used due to their better conductivity during freeze drying. Prepared aluminum dishes were placed in a -80° C freezer for overnight. The following day, small holes were made in aluminum foil covering aluminum dishes and placed in freeze drier for 72 hours. After freeze drying, samples were ground in a coffee grinder to a fine powder.

For digestion, 0.5 g of ground samples (3 replicates) were weighed in 50 ml of centrifuge tube, 10 ml (pH 2.0) of a mixed solution of 140 mM NaCl and 5 mM KCl was added, mixed and adjusted pH to 2.0 with 0.1 M HCl. Samples were treated with pepsin, incubated and rocked for 1 hour followed by adjusting pH to 5.5-6.0 with 0.1 M $NaHCO_3$. Then samples were treated with 2.5 ml of pancreatic bile solution. The pH of solutions were adjusted to 6.9-7.0 with 0.1 M $NaHCO_3$. Pepsin and pancreatic-bile solutions were purified by cation exchange resin (Chelex® 100, Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A).

Caco-2 cells were cultured in six well plates, and upper chamber was formed by placing inserts, which had 15 kDa dialysis membrane at the bottom. The dialysis membrane prevented damage to cells caused by digestive enzymes. After digestion, 1.5 ml of peptic and pancreatic digest was fed to Caco-2 cells through dialysis membrane inserts. Then the cells were placed in an incubator and rocked gently for 2 hours. During incubation, the soluble iron in the digest is expected to diffuse into the lower compartment to the Caco-2 cell monolayer (Glahn et al., 1998). After incubation, the digested sample solution along with membrane was removed, and

the cells were placed back in the incubator without rocking for 22 hours. Finally, cells were harvested for analysis of total protein using a colorimetric assay (DC™ 174 Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) and for analysis of ferritin using an immunoradiometric assay (Fer-Iron II, Ramco Laboratories, Inc., Stafford, TX, USA). Ferritin was expressed per unit of cell proteins i.e. ng ferritin mg^{-1} of cell protein. Iron bioavailability (ng ferritin mg^{-1} of protein) of samples from different experimental runs was standardized by a standard pea control (CDC Bronco) sample included in each run.

3.4.6 Measurement of carotenoid concentration using High-Performance Liquid Chromatography (HPLC)

Approximately 100 mg of ground sample was placed into a 2 ml Eppendorf tube. About 400 µl of (1:1 of methanol and DCM (dichloromethane) with 0.1 % BHT (butylated hydroxytoluene) was added to extract carotenoids. The solution was centrifuged for 15 min at 11,000 rpm, after briefly vortexting. The supernatant was decanted into a 2 ml Eppendorf tube and 400 µl of 100 % acetonitrate + 0.1 % BHT and centrifuged at 11,000 rpm for 5 min. The supernatant was then filtered through a disc filter mounted on a 1 ml syringe into the inserts in the amber glass vials and capped the vial for HPLC analysis. For chromatographic separation of carotenoids, a Prodigy 5 µm (250 x 4.60 mm) column was used with the mobile phase 58:20:22 acetonitrile/ dichloromethane/ methanol flowing at 0.8 ml/minute. The injection volume for each sample was 100 µl. The total run time for each sample was 40 minutes. Compound detection was achieved using a photodiode array detector monitoring at a 450 nm wavelength.

Standard calibration. Standards of lutein and violaxanthin (ChromaDex, Irvine, CA, USA), zeaxanthin and β-carotene (95 % purity) (Sigma-Aldrich Canada, Oakville, ON) were used to construct linear standard curves by injecting 2-40 ng (violaxanthin) or 4 -100 ng (others). Standard extraction solvents were initially premixed with 0.1 % butylated hydroxytoluene (BHT)

in dichloromethane with methanol (DCM:MeOH with 3:1) and acetonitrile (100 %). These solvents were mixed as v/v (3:1) for β-carotene and v/v (1:1) for others. All the stock solutions of chemical references were stored at -80°C. Chromatographic peaks were identified by comparing retention times and absorbance spectra to those of standards. A peak was identified as a putative carotenoid if characteristic triple maxima were observed in the absorbance spectrum and retention time was 3.9, 4.6, 5.5 and 22.0 min for violaxanthin, lutein, zeaxanthin and β-carotene, respectively (Figure 3.2). All carotenoids were detected at 450 nm, the maximum absorbance for lutein.

3.4.7 Statistics

Analysis of variance for phytate, iron concentration, carotenoid concentrations and iron bioavailability in PR-07 categorized RILs (10 lines each of yellow cotyledon-high iron concentration (YH), yellow cotyledon-low iron concentration (YL), green cotyledon-high iron concentration (GH), and green cotyledon-low iron concentration (GL)), parents (Carrera and CDC Striker) and check CDC Bronco were conducted using PROC GLM in SAS 9.3. Least square means (lsmeans) of different constituent's concentration for categorized PR-07 RILs, parents and check variety were compared with Tukey-Kramer post-hoc method. PROC CORR was run with all measured variables to find correlation among them. A multiple regression was run to predict iron bioavailability from phytate, iron, lutein, violaxanthin, zeaxanthin and β-carotene concentration using PROG REG. The assumptions of linearity, independence of errors, homoscedasticity, unusual points and normality of residuals were met.

3.5 Results

3.5.1 Results from QTL Analysis

A QTL analysis was conducted based on the GoldenGate data for iron concentration using MapQTL 5.0. The two QTL identified by Liu (2012) were confirmed (Table 3.2). Next, QTL analysis was conducted with a dense linkage map developed from GoldenGate and GBS (genotyping by sequencing) SNP markers. The QTL identified using this approach had higher LOD scores and greater proportion of phenotypic variance explanation (Table 3.2). The QTL found with GoldenGate data were confirmed on LG3 and LG4 explaining 23 % and 11 % of the phenotypic variation (Table 3.2 and Figure 3.1). The QTL on LG7 found in the GBS map was located near the QTL on LG7 identified in the GoldenGate map.

Table 3.2 Three QTL associated with iron concentration from all location-years for PR-07 RILs using the linkage maps from GoldenGate, GBS, and GoldenGate plus GBS.

QTL	Linkage Group	GoldenGate markers	GBS markers	GoldenGate plus GBS markers
QTL.Fe1	LG3	PsC6940p137 PsC7000p195 PsC3270p439 PsC18899p425 PsC12810p262 PsC17710p220	Sc12908_28149 Sc3703_405996	Sc1203_101100 PsC17710p220
LOD Score		5.9	7.3	8.1
% PV Explanation		16.8	21.2	23.0
QTL.Fe2	LG4	PsC13132p622 PsC8027p461 PsC4833p179 PsC8649p435 PsC8715p209 PsC1957p341 PsC9619p120	Sc1661_151408 Sc8496_135459	Sc9618_162688 PsC4833p179
LOD Score		4.0	3.6	4.5
% PV Explanation		10.8	9.2	11.0
QTL.Fe3	LG7	PsC8364p641 PsC14542p181 PsC21074p390 PsC17565p62	Sc2559_48386 Sc8499_18105	Sc2559_48386 PsC908p622
LOD Score		4.4	6.0	6.6
% PV Explanation		13.4	16.6	17.0
Total phenotypic explanation		40.9	47.0	51.0

LG: Linkage Group; Ps: *Pisum sativum*; C: contig; p: position; Sc: Scaffold; Number followed by underscore sign (_): Nucleotide position; LOD Score: LOD score from composite interval mapping with QTL Cartographer; PV: phenotypic variance

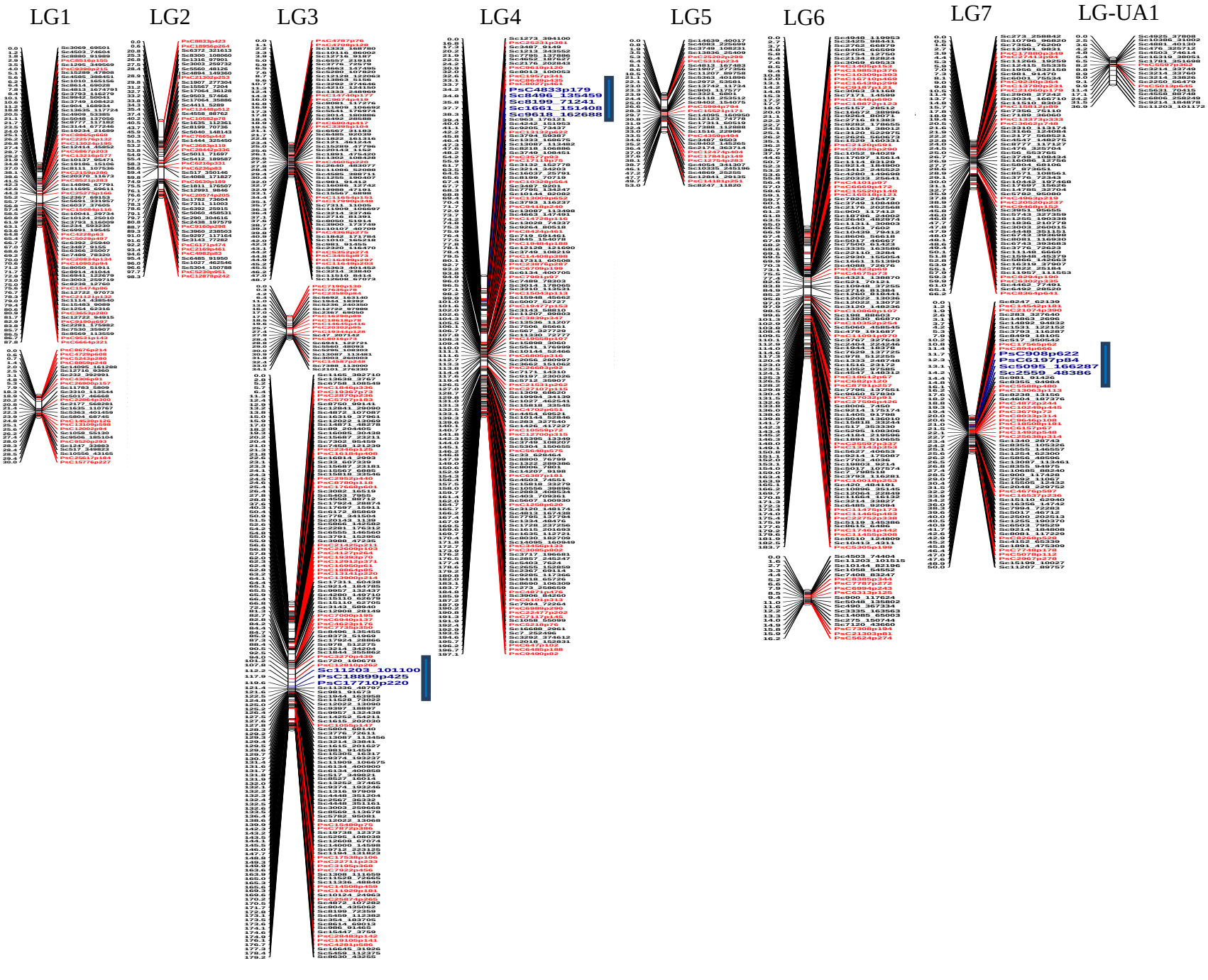

Figure 3.1 Linkage groups from PR-07 linkage map (891 loci) developed from GBS (~3200 loci, black) and GoldenGate SNPs (383, red). Blue markers indicate QTL for iron concentration.

3.5.2 Statistical analysis of phytate, iron and carotenoid (lutein, violaxanthin, zeaxanthin and β-carotene) concentration and iron bioavailability in selected PR-07 RILs

Analysis of variance for phytate, iron concentration, carotenoid concentrations and iron bioavailability in PR-07 categorized RILs, parents (Carrera and CDC Striker) and check CDC Bronco (Warkentin et al., 2005) showed significant differences for all constituents except iron bioavailability (Table 3.3).

Table 3.3 Mean squares of combined ANOVA, R-square, range and mean of different constituents in pea seeds of PR-07 RILs, parents (Carrera and CDC Striker) and check variety (CDC Bronco).

Source	DF	F Value	R-Square	Range	Mean
Phytate (mg g^{-1})	6	14.5*	0.67	0.9-2.6	1.9
Iron (mg kg^{-1})	6	19.5*	0.74	37.2-53.8	46.9
Violaxanthin (mg kg^{-1})	6	7.2*	0.51	0.2-1.4	0.6
Lutein (mg kg^{-1})	6	6.8*	0.49	5.8-11.3	8.2
Zeaxanthin (mg kg^{-1})	6	5.9*	0.46	0.0-0.7	0.2
β-carotene (mg kg^{-1})	6	20.1*	0.74	0.2-0.3	0.2
TC (mg kg^{-1})	6	8.5*	0.55	6.2-13.6	9.2
FeBIO (ng ferritin mg^{-1} of protein)	6	1.0^{ns}	0.12	5.1-12.9	8.3

TC: total carotenoids; FeBIO: iron bioavailability; DF: degree of freedom = 6: as 7 lines/ varieties (4 categorized RILs, 2 parents and CDC Bronco) were used); *: significant at <0.05 level; ns: not significant

Table 3.4 Concentration of iron, phytate, and carotenoids in seeds from PR-07 categorized RILs, parents and check variety.

Line/ Variety	Number of lines	CC	Phytate	Fe	Lut	Vio	Zea	β-C	TC*	FeBIO
			mg g^{-1}	mg kg^{-1}	mg kg^{-1}					ng ferritin mg^{-1} of protein
PR-07 GH	10	Green	1.4^{b}	50.8^{a}	9.3^{a}	1.0^{a}	0.2^{a}	0.6^{a}	11.1^{a}	8.4^{a}
PR-07 YH	10	Yellow	1.5^{b}	51.2^{a}	8.8^{a}	0.5^{b}	0.2^{a}	0.2^{b}	9.7^{a}	9.1^{a}
PR-07 GL	10	Green	2.0^{a}	42.4^{b}	8.5^{a}	0.7^{ab}	0.2^{a}	0.4^{b}	9.8^{a}	7.3^{a}
PR-07 YL	10	Yellow	2.2^{a}	44.3^{b}	6.8^{b}	0.4^{b}	0.2^{a}	0.1^{b}	7.5^{b}	8.4^{a}
Carrera	2	Yellow	1.9^{ab}	47.3^{ab}	4.6^{b}	0.2^{b}	0.1^{b}	0.0^{b}	4.9^{b}	10.7^{a}
CDC Striker	2	Green	1.8^{ab}	44.5^{ab}	7.2^{ab}	0.2^{b}	0.2^{ab}	0.0^{b}	7.5^{ab}	8.8^{a}
CDC Bronco	2	Yellow	2.6^{a}	45.3^{ab}	7.9^{ab}	0.7^{ab}	0.2^{b}	0.0^{b}	8.7^{ab}	8.3^{a}
CV			15.9	4.9	13.7	35.7	12.9	58.8	14.6	23.4

GH: green cotyledon with high iron; YH: yellow cotyledon with high iron; GL: green cotyledon with low iron; YL: yellow cotyledon with low iron; CC: cotyledon color; Lut: lutein; Vio: violaxanthin; Zea: zeaxanthin; β-C: β-Carotene ; CV: coefficient of variation; within a column, different letters indicate significant differences at $p < 0.05$; TC: total carotenoids; *: total carotenoids are sum of four carotenoids (lutein, violaxanthin, zeaxanthin and β-carotene)

3.5.2.1 Phytate concentration

GH (1.4 mg g^{-1}) and YH (1.5 mg g^{-1}) had lower mean phytate concentration than GL (2.0 mg g^{-1}) and YL (2.2 mg g^{-1}). Carrera (1.9 mg g^{-1}) and CDC Striker (1.8 mg g^{-1}) were not statistically different for phytate concentration. CDC Bronco (2.6 mg g^{-1}) had highest phytate concentration among these samples (Table 3.4).

3.5.2.2 Iron concentration

PR-07 RILs which were classified in the high iron categories had significantly greater iron concentration than those in the low iron categories. GH (50.8 mg kg^{-1}) and YH (51.2 mg kg^{-1}) had significantly higher iron concentration than GL (42.4 mg kg^{-1}) and YL (44.3 mg kg^{-1}). Carrera (47.3 mg kg^{-1}), CDC Striker (44.5 mg kg^{-1}) and CDC Bronco (45.3 mg kg^{-1}) did not differ significantly for iron concentration (Table 3.4).

3.5.2.3 Carotenoids concentration

The YL category of PR-07 RILs had lower lutein concentration than the other three categories. Green cotyledon RILs had greater violaxanthin and β-carotene concentration than yellow cotyledon RILs (Figure 3.2). There was no statistical difference among categorized RILs for zeaxanthin concentration. GH, GL, YH had greater total carotenoid concentration than the YL category (Table 3.4).

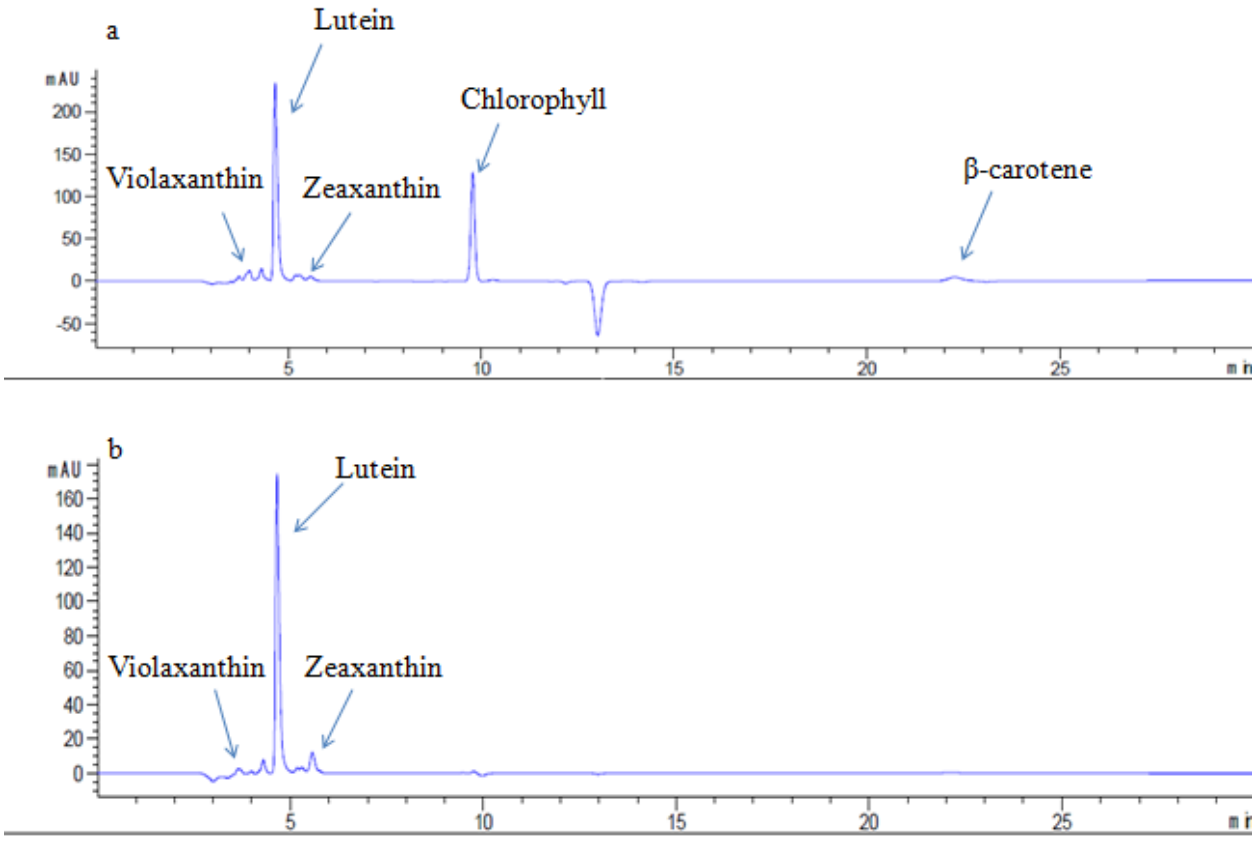

Figure 3.2 Typical chromatogram of carotenoid profile of (a) green cotyledon line (PR-07-35), and (b) yellow cotyledon line (PR-07-141).

3.5.2.4 Iron bioavailability

Although the categorized PR-07 RILs (10 RILs each), parents and CDC Bronco showed statistically significant difference for all other measured constituents, they did not differ significantly for iron bioavailability (Table 3.4).

3.5.3 Correlation of several constituents with iron bioavailability

Correlation analysis showed a significant positive correlation of iron concentration with iron bioavailability (Table 3.5). Correlations between iron bioavailability and the other constituents (phytate, lutein, violaxanthin, β-carotene, zeaxanthin, and total carotenoids concentration) were not significant.

Table 3.5 Correlation matrix of violaxanthin, lutein, zeaxanthin, β-carotene, total carotenoids, iron, phytate, molar ratio of PA:Fe and iron bioavailability in selected PR-07 RILs

Variables	Violaxanthin	Lutein	Zeaxanthin	β-C	TC[a]	Iron	Phytate	PA:Fe	FeBIO
Violaxanthin	1.00	0.70*	0.13^{ns}	0.79*	0.82*	0.26^{ns}	-0.39*	-0.39*	-0.04^{ns}
Lutein		1.00	0.23^{ns}	0.56*	0.98*	0.34*	-0.46*	-0.47*	0.08^{ns}
Zeaxanthin			1.00	-0.18^{ns}	0.19^{ns}	0.20^{ns}	0.04^{ns}	-0.05^{ns}	-0.14^{ns}
β-C				1.00	0.70*	0.23^{ns}	-0.36*	-0.33*	-0.02^{ns}
TC[a]					1.00	0.34*	-0.47*	-0.48*	0.05^{ns}
Iron						1.00	-0.62*	-0.79*	0.38*
Phytate							1.00	0.96*	-0.26^{ns}
PA:Fe								1.00	-0.29^{ns}
FeBIO									1.00

β-C: β-carotene; TC: total carotenoids; [a]: sum of four carotenoids (violaxanthin, lutein, zeaxanthin and β-carotene) measured; PA:Fe: molar ratio of phytic acid to iron; FeBIO: iron bioavailability; *: significant at 0.05 level; ns: not significant.

3.5.4 Multiple regression analysis

Analysis of variance showed that a regression model of iron bioavailability from predictor variables (β-carotene, zeaxanthin, iron, phytate, lutein concentration) resulted in a statistically significant prediction of iron bioavailability (Table 3.6).

Table 3.6 Analysis of variance[a] from multiple regression analysis for iron bioavailability in PR-07 categorized RILs

Model	Sum of Squares	DF	Mean Square	F	Sig.	R Square
Regression	34.9	5	7.0	2.46	0.051[b]	0.25
Residual	102.3	36	2.8			
Total	137.2	41				

[a]: Dependent Variable: iron bioavailability; [b]: Predictors: (constant), β-carotene, zeaxanthin, iron, phytate, lutein concentration; DF: degree of freedom = 5 (1 dependent and 5 independent variables were used)

A multiple regression was run to predict iron bioavailability from phytate, iron, lutein, zeaxanthin and β-carotene concentration. The assumptions of linearity, independence of errors, homoscedasticity, unusual points and normality of residuals were met. These variables significantly predicted iron bioavailability, F (5, 36) = 2.46, p = 0.051, R^2 = 0.25. Iron concentration had the highest standardized beta coefficient (0.419, p = 0.03), indicating that iron concentration had the greatest association with iron bioavailability (Table 3.7).

Table 3.7 Estimation of model coefficients from multiple regression analysis in PR-07 RILs

Variable	B	SE_B	ß
Intercept	5.181	5.046	
Phytate	-0.042	0.880	-0.01
Iron	0.175	0.078	0.419*
Lutein	0.172	0.263	0.145
Zeaxanthin	-29.720	14.342	-0.359
β-carotene	-1.760	1.345	-0.247

*: $p < 0.05$; B = standardized regression coefficient; SE_B = standard error of the coefficient; ß = standardized coefficient

3.6 Discussion

To produce an iron-dense field pea variety, identification of markers that can help in selection of high/low iron density genotypes will be beneficial (Collard et al., 2005). We have confirmed three QTL associated with iron concentration in a pea recombinant inbred line population PR-07 (Carrera X CDC Striker). The QTL were located on a GoldenGate linkage map (with 383 loci) and further finely mapped on a GBS linkage map (3131 loci). Using the GoldenGate linkage map, three iron concentration loci were mapped on LG3, LG4 and LG7, which individually explained 11 to 17 % of the phenotypic variance and in total explained 41 % (Table 3.2). Using the GBS linkage map, the three QTL associated with iron concentration were also located on LG3, LG4 and LG7 with LOD scores of 8.1, 4.5 and 6.6, respectively (Table 3.2), explaining 23 %, 11 %, 17 % individually and 51 % in total. In the model legume *Medicago truncatula* L., QTL associated with iron concentration were identified on chromosome 7 and 8 using a linkage map generated from microsatellite (SSR) markers (Sankaran et al., 2009), with LOD scores of 3.3 and 3.9, respectively, explaining 21.9 % of the phenotypic variance. Chromosome 7 and 8 of *Medicago truncatula* L. correspond with pea LG4 and LG5 (Sindhu et al., 2014). In the present study, the three identified QTLs explained 51 % of the phenotypic variance in total (Table 3.2), which is greater than the phenotypic variance (21.6 %) explained by two QTLs identified in *Medicago truncatula* (Sankaran et al., 2009). Higher phenotypic variance explanation can be attributed to greater marker density and quality. Moreover about 3200 SNP markers reduced the QTL map intervals in the present study as compared to 367 SSR markers used while performing QTL mapping in *Medicago truncatula.*

QTLs for iron concentration on LG3 and LG4 were identified using the GoldenGate array linkage map, and further finely mapped and confirmed with the GBS map with higher LOD score. The QTL on LG7 from the GBS linkage map had an insignificant LOD score on the

Goldengate linkage map. A similar phenomenon was also seen in barley when Goldengate and GBS linkage maps were compared, in that some marker arrangements were shifted (Wendler et al., 2014). Marker locations may rearrange when more markers are added to linkage maps (Wendler et al., 2014). Mendel (1866) first described the mutation *i* for green cotyledon color, which was located on LG1 (McCallum et al., 1997) and thus not linked to the three QTL for iron concentration described in this research.

This study included the evaluation of iron bioavailability in PR-07 RILs. Forty PR-07 RILs were selected which were contrasting for cotyledon color and iron concentration (Table 3.1). The selected PR-07 lines were categorized in high and low iron categories according to their iron concentration. Among the lines within each category, the variability in iron concentration was low (Table 3.4). The FeBIO assay has a moderate amount of inherent variation (Volpe, 2008). Iron concentration ranged from 37-54 mg kg^{-1} and iron bioavailability ranged from 5-13 ng ferritin mg^{-1} of protein (Table 3.4). Similarly, common bean varieties with relatively low variability in iron concentration (48-74 mg kg^{-1}) had moderate variation in iron bioavailability (1.1-11.5 ng ferritin mg^{-1} of protein) using the Caco-2 cell culture assay (Ariza-Neito et al., 2007). The selected RILs differed significantly for iron concentration, but no significant differences were detected for iron bioavailability (Table 3.4). Although iron bioavailability in high and low iron concentration lines was similar, a significant modest correlation (r=0.38) between iron concentration and iron bioavailability was observed (Table 3.5). Strong correlation (r=0.89) of iron concentration and iron bioavailability was reported in common bean varieties, which differed in iron concentration (Welch et al., 2000). Moreover, when common bean lines were selected with high iron concentration, which reduced the polyphenols to iron molar ratio, an increase in iron bioavailability was observed (Petry et al., 2010). However, in a study of rice

varieties, no correlation was observed between iron concentration and iron bioavailability (Glahn et al., 2002).

A significant negative correlation (r = -0.62) was detected between iron and phytate concentration in selected PR-07 RILs (Table 3.5). Ren et al. (2007) reported that rice lines with a 47 % reduction in phytate concentration also had 71 % greater iron concentration than the normal phytate parent. In the present study, green (30 %) and yellow (32 %) cotyledon lines with relatively lower phytate concentration had 20 % and 16 % more iron concentration, respectively. However, no correlation was observed between iron and phytate concentration in common bean genotypes (Welch et al., 2000). Reduction of phytate concentration was observed to enhance iron bioavailability and nutritional value of foods (Hurrell et al., 1992; Thacker et al., 2013). A 50 % reduction in phytic acid concentration in *lpa* pea lines enhanced iron bioavailability up to 2 times compared to the normal phytate cultivar (Liu et al., 2014). In the current study, although the lines with high iron concentration had relatively low phytate concentration, which might be an additive benefit for iron bioavailability, no significant differences were detected in high vs. low iron categories (Table 3.4).

Carotenoid concentrations in PR-07 RILs (Table 3.4) were lower than the concentrations found in pea cultivars described by Ashokkumar et al. (2014; in Table 2). This may be attributed to the fact that the carotenoid extraction in PR-07 RILs samples was conducted 3 years after sample harvest, while the carotenoid extraction of samples in Ashokkumar et al. (2014) was conducted shortly after harvesting. Carotenoid degradation is known to occur with time and conditions of storage (Hidalgo and Brandolini, 2008; Ferrante et al., 2004).

Lutein concentration was highest among all carotenoids measured and it ranged from 5.8 to 11.3 mg kg^{-1} (Table 3.4). Lutein was the dominant seed and hull carotenoid and consistently

observed in peas from previous studies (Ashokkumar et al., 2014; Maiani et al., 2009; Marles et al., 2013; Edelenbos et al., 2001; McCallum et al., 1997). Zeaxanthin and violaxanthin concentrations in selected RILs were comparable to that found by Ashokkumar et al (2014). β-carotene concentration was the lowest among all carotenoids measured and found to be higher in green than yellow cotyledon pea lines (Table 3.4), with a similar trend reported previously (Ashokkumar et al., 2014). Marles et al. (2013) observed trace amount of β-carotene in the hull of green cotyledon pea cultivar Orb. β-carotene varied from 0.13-0.33 mg kg^{-1} in three yellow cotyledon pea cultivars (Nameskéri, 2006). Supplementation of cereal-based diets with carotenoid compounds (lutein, zeaxanthin and β-carotene) increased iron absorption in human adults (García-Casal., 2006; García-Casal et al, 1998). In the current study, no significant effect of any carotenoid compound on iron bioavailability was observed.

In this study, three QTL associated with iron concentration were finely mapped on the PR-07 linkage map using GBS technology. This resulted in higher LOD scores and greater phenotypic variance explanation compared to the previously available GoldenGate array linkage map. Iron concentration in PR-07 RILs was positively correlated with iron bioavailability suggesting a nutritional benefit of selecting pea lines with increased iron concentration.

CHAPTER 4

IRON BIOAVAILABILITY IN PEA LINES CONTRASTING IN COTYLEDON COLOUR AND PHYTIC ACID PHOSPHORUS CONCENTRATION

4.1 Introduction

Breeding lines contrasting in cotyledon colour and phytic acid phosphorus (PA-P) (i.e. phytate) concentration were available from the CDC field pea breeding program. Cross 4802 (1-2347-144/CDC 2235-4) and cross 4803 (1-150-81/CDC 2336-1) were made in winter 2011. Green cotyledon variety CDC 2235-4 was later registered as CDC Raezer (Warkentin et al., 2014). Green cotyledon variety CDC 2336-1 was later registered as CDC Limerick (Warkentin et al., 2014). 1-2347-144 and 1-150-81 are low phytate lines with yellow cotyledon seeds (Warkentin et al., 2012). No selections were made in the F_1 generation. Selection in the F_2 generation was based on seed traits (size, shape, cotyledon colour), but specific data were not recorded. Selection in the F_3 was based on agronomic performance and seed appearance. In 2013, 10 F_4 lines from cross 4802 and 4 F_4 lines from cross 4803 were grown in field trials with one replication at Rosthern and one replication at Meath Park, Saskatchewan.

4.2 Hypotheses

1. Pea seeds with greater carotenoid concentration will have greater iron bioavailability.
2. Low phytate concentration and high carotenoid concentration in pea seeds will have additive benefits for iron bioavailability.

4.3 Objective

1. To compare iron bioavailability in pea lines contrasting in phytate concentration and cotyledon color.

4.2 Materials and Methods

4.2.1 Plant Material

Twenty-four F_5 seeds from each of the 10 F_4 lines from cross 4802 and from each of the 4 F_4 lines from 4803 were analyzed for inorganic phosphorus (P_i) concentration using Chen's reagent method. CDC McGwire, a normal phytate barley variety, HB379, a low phytate barley variety, CDC Bronco (Warkentin et al., 2005), a normal phytate pea, and 1-150-81 and 1-2347-144 the low phytate pea lines were used as controls. Seventy six percent of the seeds from line 4802-8 and 71 % of the seeds from 4803-4 had high concentration of inorganic phosphorus. Both lines were segregating for cotyledon color as well. One hundred yellow and 180 green cotyledon seeds from each of these two lines were analyzed for inorganic phosphorus (P_i) concentration by cutting a small cotyledonary portion of individual F_5 seeds, and the same seeds were used for multiplication of seeds in the greenhouse. Based on previous research, higher inorganic phosphorus concentration was associated with lower concentration of phytate in pea seeds (Warkentin et al., 2012). Based on P_i, seeds were categorized into four categories: green color low phytate (GL), green color normal phytate (GN), yellow color low phytate (YL), and yellow color normal phytate (YN). Seeds were classified as normal phytate when inorganic phosphorus concentration was similar to that of CDC Bronco; seeds were classified as low phytate when inorganic phosphorus concentration was similar to the two low phytate (1-2347-144 and 1-150-81) lines.

The rest of the F_5 seed with embryo axis intact were planted in the Agriculture Greenhouse in January 2014 for multiplication of seeds. These included 25 green cotyledon low phytate (GL), 20 green cotyledon normal phytate (GN), 25 yellow cotyledon low phytate (YL), and 25 yellow cotyledon normal phytate (YN) seeds from each of 4802-8 and 4803-4 lines; these were referred to as ‘categorized sub-lines’. In addition, control CDC Bronco and the two *lpa* lines (1-

150-81 and 1-2347-144) were planted. Flowering and pod filling started in the second week of March. Fertilizer (Plant-Prod 20:20:20 Classic) was applied with water every week and watered as necessary. Forty days after planting, a control release type-40 fertilizer was applied, which released nutrients gradually. Sub-lines were harvested in the first week of May, 2014. About 60 to 100 F_6 seeds were obtained from each sub-line.

4.2.2 Biochemical Analysis of F_6 sub-lines from greenhouse

Twenty F_6 seeds from each sub-line were analyzed for phytate concentration to confirm their phenotype. Again a small portion of cotyledon was used to analyze inorganic phosphorus/phytate concentration using Chen's reagent method.

4.2.3 Field trial in 2014 and 2015

About 50-60 F_6 seeds (2014) and about 50-60 F_7 seeds (2015) from each of the sub-lines of 4802-8 and 4803-4 lines, comprising four phenotypic classes, i.e., green low phytate, yellow low phytate, green normal phytate, yellow normal phytate, along with parents and check varieties (i.e. 1-2347-144, 1-150-81, CDC Limerick and CDC Raezer and CDC Bronco) were planted in the Sutherland (Saskatoon) field in a single replicate trial in 1 m^2 micro-plots.

4.2.4 Phenotypic data from 2014 and 2015 field trials

Typical agronomic assessments for field pea were taken. Measurement of emergence was recorded 8 weeks after seeding and converted to percentage. Days to flower were calculated at the stage when 10 % of plants within microplot had at least one flower. Mycosphaerella blight ratings (1 to 9) were recorded at 10 days after flowering and successively after 2 weeks for three readings. Lodging rating (from 1 to 9) was recorded at physiological maturity. Plant height was measured at complete pod set. Other measurements were days to maturity, seed weight and grain yield. After harvest, samples were analyzed for iron bioavailability, phytate, inorganic phosphorus, carotenoids, and iron concentration.

4.2.5 Inorganic phosphorus assays of samples harvested in 2014

Inorganic phosphorus concentration was measured for harvested F_7 seeds from 4802-8 and 4803-4 sub-lines. Twelve seeds from each categorized sub-line were tested along with parent checks (1-2347-144, CDC Raezer, 1-150-81, CDC Limerick) and CDC Bronco.

Inorganic phosphorus concentration. The modified Chen's reagent method (Chen et al., 1956) was used to assess the inorganic phosphorus concentrations in field pea samples. These tests were conducted in the Plant Sciences Department, University of Saskatchewan. Chen's reagent consists of 1 volume of 6N H_2SO_4, 1 volume of 2.5 % ammonium molybdate, 1 volume of 10 % ascorbic acid (which is stored at 4°C) and 2 volumes of double distilled water. Fifty mg of 0.5 mm ground sample was placed in a tube for each sample and 1 ml of 0.4 M HCl was added. The aliquots were incubated overnight at 4°C then vortexed for 10 seconds. After further 30 minutes incubation, 10 µl extracts were placed in microtitre plate wells with 90 µl of double distilled H_2O and 100 µl of Chen's reagent. Standards were prepared as explained in the following table.

1 mM K_2HPO_4 (µL)	0.4 M HCl (µL)	Double Distilled Water (µL)
0	10	90
10	10	80
20	10	70
30	10	60
40	10	50
50	10	40

The mixtures were incubated for two hours to develop before reading the absorbance values on microplate reader at 655 nm. A standard curve was calculated based on the concentration of standards and their absorbance values. The absorbance value for each well was converted to µl of 1 mM K_2HPO_4. Seeds with normal-phytate concentration had produced

colorless solutions, while low-phytate seeds produced blue solutions depending on the amount of inorganic phosphorus.

4.2.6 Determination of iron concentration of field pea sample

The iron concentration of field pea varieties was measured using atomic absorption spectroscopy (AAS) in the Plant Sciences Department, University of Saskatchewan as described in Chapter 3.

4.2.7 Determination of phytate concentration of field pea samples

Harvested F_7 seeds with four technical repeats were analyzed for phytate concentration using Wade's reagent method (Gao et al., 2007) as described in 3.4.3. These tests were conducted in the Plant Sciences Department, University of Saskatchewan.

4.2.8 Total phosphorus

Total P in cotyledons was extracted by the wet ashing method in (Raboy et al. 2000). These tests were conducted in the Plant Sciences Department, University of Saskatchewan. Fifty mg of ground sample was incubated with 1 ml of concentrated (18.4 M) H_2SO_4 overnight in a fume hood at room temperature. Two hundred µl of 30 % (v/v) H_2O_2 was added and the samples were incubated in a heating block between 220 and 250°C for 30 min. Samples were removed and allowed to cool at room temperature for 15 min. This cycle of heating (with H_2O_2) and cooling was repeated until the sample became clear. The volume of the sample was adjusted to 6.25 ml with ddH_2O. The total extractable P was determined with spectrophotometer using the method of Chen et al. (1956) as described above.

4.2.9 Caco-2 cell culture study

This technique was developed in the laboratory of Dr. Raymond Glahn at USDA-ARS, Cornell University, Ithaca, New York. Caco-2 studies on pea samples were conducted in Dr. Glahn's laboratory as described in Chapter 3.

Fifteen sub-lines from each cotyledon color/phytate concentration category were randomly selected for measuring iron bioavailability. Not all sub-lines could be tested due to limitations in laboratory capacity. Along with these sub-lines, 5 samples of each parent (1-2347-144 and CDC Raezer) and 7 samples of CDC Bronco (control) were chosen for iron bioavailability measurement.

4.2.10 Measurement of carotenoid concentration using High- Performance Liquid Chromatography (HPLC)

Concentrations of four different carotenoids (lutein, violaxanthin, zeaxanthin and β-carotene) were measured using HPLC. These tests were conducted in the Plant Sciences Department, University of Saskatchewan as described in Chapter 3.

4.2.11 Statistics

Analysis of variance for inorganic phosphorus, phytate, iron, and carotenoid concentration in 4802-8 and 4803-4 categorized sub-lines (green cotyledon low phytate concentration (GL), and green cotyledon normal phytate concentration (GN), yellow cotyledon low phytate concentration (YL)), parents (1-2347-144 and CDC Raezer) and CDC Bronco were conducted using PROC GLM in SAS 9.3. Least square means of iron bioavailability for categorized 4802-8 and 4803-4 sub-lines, parents and check variety were compared using the Tukey-Kramer post-hoc method. PROC CORR was run with all measured variables to find correlation among them in both sub-lines. A multiple regression was run to predict iron bioavailability from phytate, iron, lutein, violaxanthin, zeaxanthin and β-carotene concentration using PROG REG. The assumptions of linearity, independence of errors, homoscedasticity, unusual points and normality of residuals were met.

4.3 Results

4.3.1 Results from greenhouse and field trial 2014

Phytate and inorganic phosphorus (P_i) concentrations were measured for harvested F_7 seeds from 4802-8 and 4803-4 sub-lines (GL, GN and YL). Twelve seeds from each sub-line were tested along with parent checks (1-2347-144, CDC Raezer, 1-150-81, CDC Limerick) and CDC Bronco. Based on the above two assays, three categories (i.e. green low phytate, green normal phytate, and yellow low phytate seeds) produced results as expected based on how they were classified based on F_5 phenotype. But in the fourth category 'yellow cotyledon normal phytate', all 4803-4 sub-lines behaved as low phytate, while five normal phytate yellow cotyledon sub-lines were observed in this subset of 4802-8 (Table 4.1). The total phosphorus concentration was found to be similar among all categories of sub-lines. Agronomic characteristics of these sub-lines were measured to observe growth and development of plants in the field. These characteristics were summarized in Appendix 1 and 2.

Table 4.1 Inorganic phosphorus (mg g^{-1}), phytic acid phosphorus (PA-P) (mg g^{-1}) and total phosphorus concentration (mg g^{-1}) of categorized sub-lines (4802-8 and 4803-4), their parents (1-2347-144. CDC Raezer, 1-150-81 and CDC Limerick) and check variety (CDC Bronco) grown in the field at Sutherland in 2014

Lines	Number of observed lines		Concentration at F_7 generation		
	F_6	F_7	P_i ($mg\ g^{-1}$)	PA-P ($mg\ g^{-1}$)	Total Phosphorus ($mg\ g^{-1}$)
4802-8 GL	21	25	1.46	1.15	2.66
4802-8 GN	20	16	0.78	2.13	2.48
4802-8 YL	22	42	1.62	1.19	2.21
4802-8 YN	25	5	1.34	2.18	2.45
4803-4 GL	23	22	1.51	1.14	2.49
4803-4 GN	19	20	0.38	2.42	2.24
4803-4 YL	25	47	1.56	0.97	2.49
4803-4 YN	22	0	n/a	n/a	n/a
CDC Bronco	10	10	0.37	2.49	2.48
1-150-81	10	10	1.39	0.96	2.38
1-2347-144	10	10	1.47	1.10	2.21
CDC Limerick	10	10	0.43	2.73	2.40
CDC Raezer	10	10	0.38	2.97	2.36

GL, green cotyledon and low phytate; GN, green cotyledon and normal phytate; YL, yellow cotyledon and low phytate; YN, yellow cotyledon and normal phytate; P_i, inorganic phosphorus; PA-P, phytate phosphorus; F_6: classification at 6th filial generation; F_7: observations at 7th filial generation.

4.3.2 Regression analysis between inorganic phosphorus and phytate concentration

The regression observed between inorganic phosphorus concentration and phytic acid phosphorus concentration was significantly negative in the case of both categorized 4802-8 ($r = 0.25$; $p < 0.001$) and 4803-4 sub-lines ($r = 0.55$; $p < 0.0001$) (Figure 4.1 and 4.2).

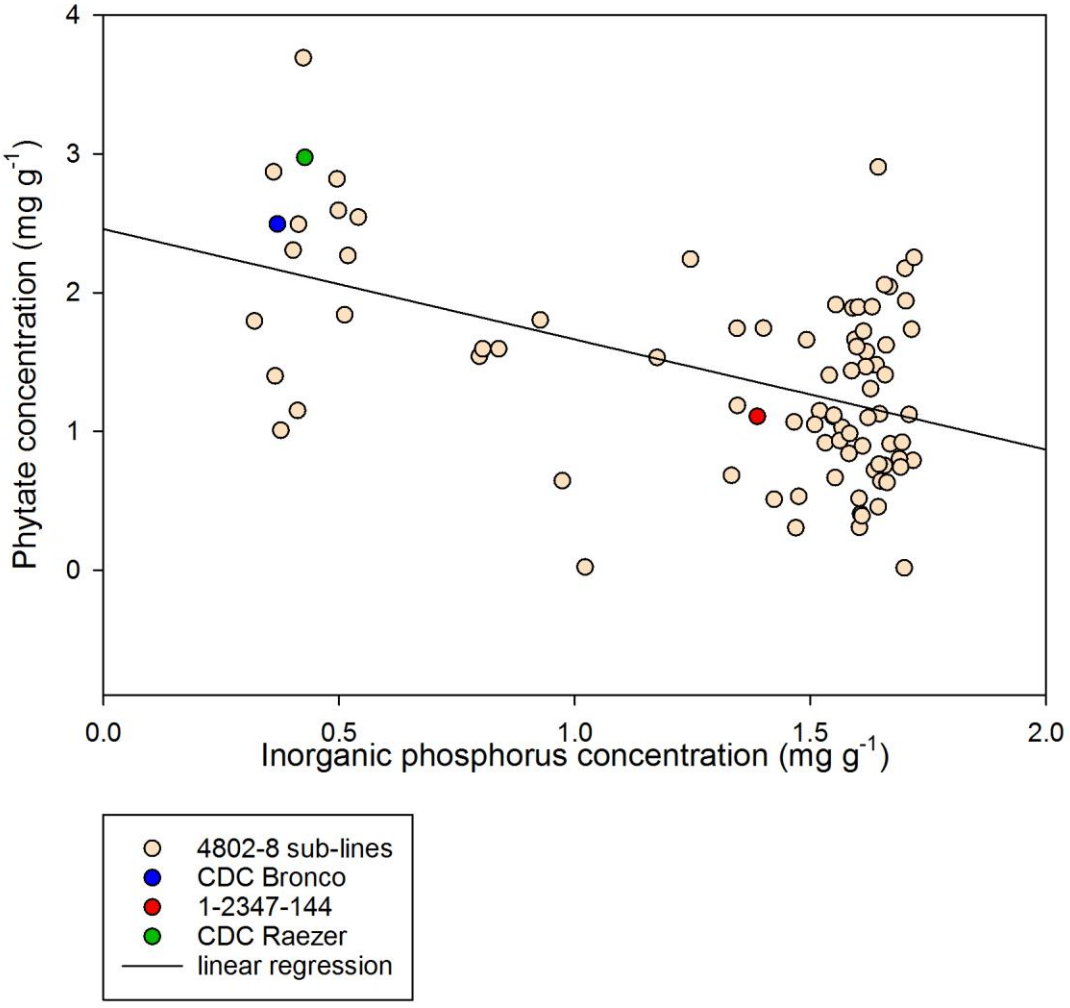

Figure 4.1 Scatterplot between the phytate (mg g^{-1}) and inorganic phosphorus (mg g^{-1}) concentrations of 4802-8 sub-lines and parents (1-2347-144 and CDC Raezer) at Sutherland in 2014.

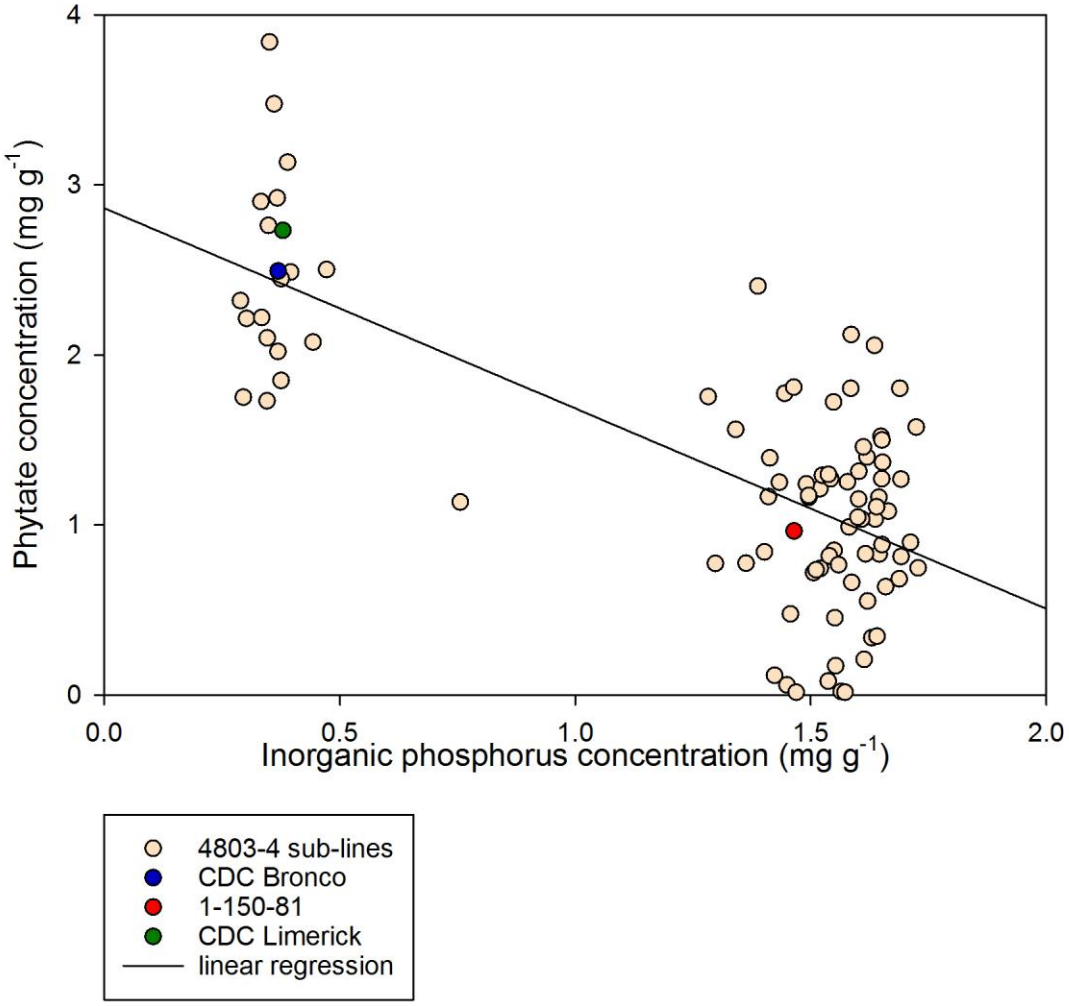

Figure 4.2 Scatterplot between the phytate (mg g^{-1}) and inorganic phosphorus (mg g^{-1}) concentrations of 4803-4 sub-lines and parents (1-150-81 and CDC Limerick) in 2014 Sutherland.

4.3.3 Analysis of 4802-8 sub-lines

4802-8 sub-lines, (1-2347-144 and CDC Raezer) and check variety (CDC Bronco) showed significant differences for all observed constituents except zeaxanthin concentration (Table 4.2).

Table 4.2 Mean squares of combined ANOVA, R-square, coefficient of variance (CV) and mean of different constituents in pea seeds of 4802-8sub-lines, parents (1-2347-144 and CDC Raezer) and check variety (CDC Bronco).

Source	DF	F-Value	R-square	Mean
P_i (mg g^{-1})	5	127.5*	0.86	1.19
Phytate (mg g^{-1})	5	26.2*	0.55	1.59
Iron (mg kg^{-1})	5	2.5*	0.10	47.75
Violaxanthin (mg kg^{-1})	5	3.0*	0.12	1.69
Lutein (mg kg^{-1})	5	11.0*	0.34	9.15
Zeaxanthin (mg kg^{-1})	5	0.31^{ns}	0.01	0.15
β-carotene (mg kg^{-1})	5	103.4*	0.83	0.27
TC (mg kg^{-1})	5	8.8*	0.29	11.30

P_i: inorganic phosphorus; TC: total carotenoids; *: significance $p<0.05$; ^{ns}: not significant; DF: degree of freedom = 5 (3 categorized sub-lines, 2 parents and CDC Bronco were analyzed)

Table 4.3 Concentration of iron, phytate, and carotenoid concentration of seeds from 4802-8 categorized sub-lines, parents (1-2347-144 and CDC Raezer) and check variety (CDC Bronco).

Sample	Lines	CC	PC	P_i	Phytate	Iron	Lut	Vio	Zea	β-C	TC*
				—(*mg g^{-1}*)—		*mg kg^{-1}*		——————*mg kg^{-1}*——————			
4802-8 GL	24	Green	Low	1.5^{a}	1.2^{c}	48.2^{ab}	10.3^{a}	2.1^{a}	0.2^{a}	0.6^{a}	13.1^{a}
4802-8 GN	17	Green	Normal	0.6^{b}	2.1^{b}	48.1^{ab}	8.2^{b}	1.4^{a}	0.2^{a}	0.6^{a}	10.4^{bc}
4802-8 YL	42	Yellow	Low	1.6^{a}	1.2^{c}	47.9^{ab}	9.1^{b}	1.8^{a}	0.2^{a}	0.0^{b}	11.0^{ab}
CDC Bronco	10	Yellow	Normal	0.4^{b}	2.5^{ab}	45.1^{b}	7.8^{b}	0.7^{a}	0.1^{a}	0.0^{b}	8.7^{c}
1-2347-144	10	Yellow	Low	1.4^{a}	1.1^{c}	47.0^{ab}	10.8^{a}	2.2^{a}	0.2^{a}	0.0^{b}	13.1^{a}
CDC Raezer	10	Green	Normal	0.4^{b}	3.0^{a}	49.0^{a}	8.1^{b}	1.2^{a}	0.2^{a}	0.6^{a}	10.1^{bc}
CV				17.4	37.3	6.0	14.7	67.6	15.7	49.8	19.6

CC: cotyledon color; PC: phytate category; P_i: inorganic phosphorus; Lut: lutein; Vio: violaxanthin; Zea: zeaxanthin; β-C: β-carotene; TC: total carotenoids; CV: coefficient of variation; Within a column, different letters indicate significant differences at $p < 0.05$, * total carotenoid concentration was calculated as sum of four individual carotenoids.

4.3.3.1 Phytate and inorganic phosphorus concentration

YL (1.6 mg g^{-1}), GL (1.5 mg g^{-1}) sub-lines and 1-2347-144 (1.4 mg g^{-1}) had greater mean inorganic phosphorus concentration than the normal phytate sub-lines and checks (Table 4.3). Lsmeans showed YL (1.2 mg g^{-1}) and GL (1.2 mg g^{-1}) sub-lines, and 1-2347-144 (1.1 mg g^{-1}) had lower mean phytate concentration than GN sub-lines (2.1 mg g^{-1}). CDC Raezer had the highest concentration of phytate (3.0 mg g^{-1}). GN sub-lines had phytate concentration (2.1 mg g^{-1}) which was not significantly different from CDC Bronco (2.5 mg g^{-1}) (Table 4.3).

4.3.3.2 Iron concentration

All 4802-8 categorized sub-lines and CDC Raezer were not statistically different from each other for iron concentration; whereas CDC Bronco had the lowest mean iron concentration of 45.1 mg kg^{-1}. Line 1-2347-144 had iron concentration which was not significantly different from 4802-8 sub-lines and CDC Bronco (Table 4.3).

4.3.3.3 Carotenoid concentrations

Among four carotenoids, lutein constituted the major portion of total carotenoids. 1-2347-144 (10.8 mg kg^{-1}) and GL (10.3 mg kg^{-1}) had greater lutein concentration, whereas, the other sub-lines along with CDC Bronco and CDC Raezer were not statistically different from each other. Similar to lutein concentration, a greater concentration of violaxanthin was found in 1-2347-144 and GL. All categorized sub-lines and check varieties were not statistically different from each other for zeaxanthin concentration. Green cotyledon sub-lines and CDC Raezer had greater concentration of β-carotene than the yellow cotyledon sub-lines, 1-2347-144 and CDC Bronco (Table 4.3).

4.3.3.5 Iron bioavailability

Categorized low phytate sub-lines showed higher iron bioavailability than normal phytate sub-lines, parents/checks and even higher than the low phytate parent (1-2347-144).

Table 4.4 Iron bioavailability (FeBIO) from 4802-8 sub-lines categorized on the basis of cotyledon color and phytate concentration, parents of 4802 cross (1-2347-144 and CDC Raezer) and check variety (CDC Bronco).

Sample	Number of lines	Cotyledon color	Phytate category	FeBIO ng ferritin mg^{-1} of protein
4802-8 GL	15	Green	Low	13.7[a]
4802-8 GN	15	Green	Normal	9.6[b]
4802-8 YL	15	Yellow	Low	14.2[a]
CDC Bronco	7	Yellow	Normal	8.2[b]
1-2347-144	5	Yellow	Low	10.9[ab]
CDC Raezer	5	Green	Normal	6.4[b]
CV				27.6

CV: coefficient of variation; Within a column, different letters indicate significant differences at $p < 0.05$.

4.3.4 Comparison of all sub-lines with CDC Bronco

CDC Bronco is a yellow cotyledon color and normal phytate variety, progenitor of two low phytate mutants (1-2347-144 and 1-150-81); therefore, CDC Bronco was used as a control in the iron bioavailability experiments. Iron bioavailability was measured from three technical repeats of 15 samples in each category (Figure 4.3). Mean values showed GN sub-lines (9.6 ng ferritin mg^{-1} of cell protein) had 1.0-1.6 times higher iron bioavailability than CDC Bronco; whereas GL (13.7 ng ferritin mg^{-1} of protein) and YL (14.2 ng ferritin mg^{-1} of protein) had up to 2.6 and 3.2 times higher iron bioavailability than CDC Bronco, respectively. Appendix 3 compares iron bioavailability between 4802-8 sub-lines, parents and CDC Bronco.

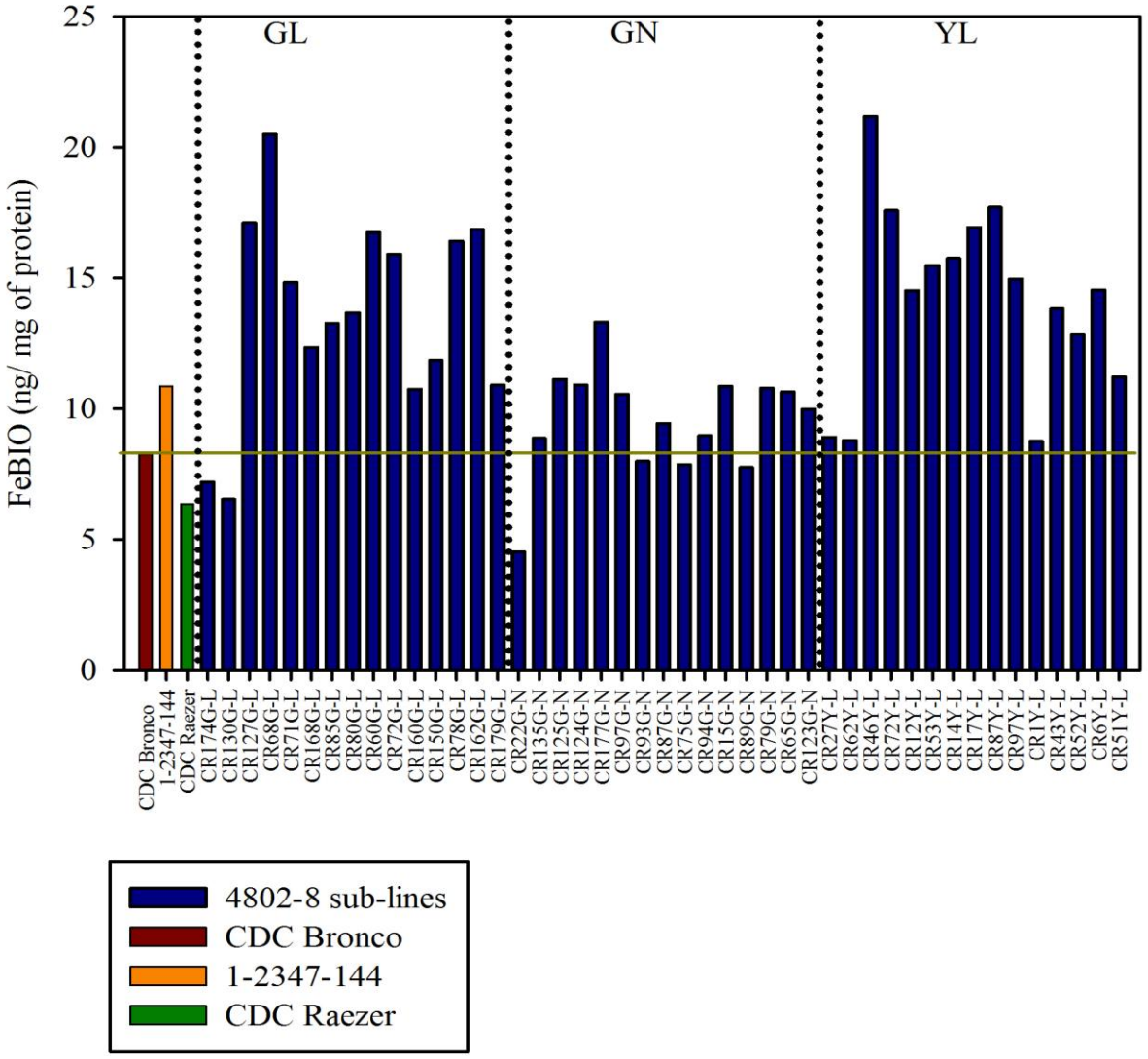

Figure 4.3 Comparison of iron bioavailability in 4802-8 sub-lines (15 samples from each) with CDC Bronco FeBIO: iron bioavailability; GL: Green cotyledon with low phytate; GN: Green cotyledon with normal phytate; YL: Yellow cotyledon with low phytate; each bar represents the mean value from three technical replicates of each sample.

The two sub-lines with the greatest (20.8 ± 0.49 ng ferritin mg^{-1} of protein) and two sub-lines with the lowest (4.4 ± 0.15 ng ferritin mg^{-1} of protein) iron bioavailability were compared for their phytate, iron, carotenoids concentration and molar ratio of PA to iron. These sub-lines differed for phytate, iron and carotenoid (lutein, violaxanthin, β-carotene) concentrations and molar ratio of PA:Fe. Phytate concentration of sub-lines with highest iron bioavailability was 72 % lower than the sub-lines with lowest iron bioavailability (Table 4.5).

Table 4.5 Phytate, iron, carotenoid (lutein, violaxanthin, zeaxanthin and β-carotene) concentrations and molar ratio of phytic acid to iron of the 4802-8 sub-lines with highest and lowest iron bioavailability.

Variable	4802-8 sub-lines mean ± SE	4802-8 sub-lines Range	2 greatest FeBIO sub-lines mean	2 lowest FeBIO sub-lines mean
Phytic acid (mg g^{-1})	1.5 ± 0.11	0.0-2.9	0.5	1.8
Iron (mg kg^{-1})	48.2 ± 0.46	42.0-55.2	43.4	47.9
Molar ratio PA:Fe	8.8 ± 0.66	0.0-19.0	3.5	4.5
Lutein (mg kg^{-1})	9.3 ± 0.29	3.9-13.2	10.3	7.8
Violaxanthin (mg kg^{-1})	1.8 ± 0.20	0.2-8.8	2.0	1.6
Zeaxanthin (mg kg^{-1})	0.2 ± 0.00	0.1-0.2	0.1	0.1
β-Carotene (mg kg^{-1})	0.4 ± 0.05	0.0-0.8	0.4	0.3
Total carotenoids (mg kg^{-1})	11.6 ± 0.45	5.5-22.0	12.8	9.8
FeBIO (ng ferritin mg^{-1} of protein)	12.5 ± 0.57	4.5-21.2	20.8	4.4

SE: standard error; FeBIO: iron bioavailability

4.3.5 Correlation of several constituents with iron bioavailability in 4802-8 sub-lines

A significant positive correlation was detected between inorganic phosphorus and lutein concentration with iron bioavailability, and a significant negative correlation of phytate concentration with iron bioavailability. Phytate concentration showed significant negative correlation with total carotenoids, lutein and violaxanthin, and a positive correlation with β-carotene concentration. Iron concentration showed positive correlation with total carotenoids and lutein concentration (Table 4.6).

Table 4.6 Correlation matrix of violaxanthin, lutein, zeaxanthin, β-carotene, total carotenoids, inorganic phosphorus, phytate, iron, molar ratio of PA:Fe and iron bioavailability in 4802-8 sub-lines

Variables	Violaxanthin	Lutein	Zeaxanthin	β-C	TC[a]	P_i	Phytate	Iron	PA:Fe	FeBIO
Violaxanthin	1.00	0.58*	-0.08^{ns}	-0.03^{ns}	0.82*	0.18^{ns}	-0.30*	0.13^{ns}	-0.30*	0.03^{ns}
Lutein		1.00	0.26^{ns}	0.32*	0.94*	0.37*	-0.33*	0.22^{ns}	-0.24^{ns}	0.41*
Zeaxanthin			1.00	0.29^{ns}	0.17^{ns}	0.02^{ns}	0.10^{ns}	0.11^{ns}	0.33*	-0.05^{ns}
β-C				1.00	0.30*	-0.42*	0.22^{ns}	0.08^{ns}	0.22^{ns}	-0.18^{ns}
TC[a]					1.00	0.27^{ns}	-0.32*	0.21^{ns}	-0.26^{ns}	0.26^{ns}
P_i						1.00	-0.55*	-0.02^{ns}	-0.50*	0.48*
Phytate							1.00	0.13^{ns}	0.71*	-0.34*
Iron								1.00	0.12^{ns}	-0.15^{ns}
PA:Fe									1.00	-0.18^{ns}
FeBIO										1.00

β-C: β-carotene; TC: total carotenoids; [a]: sum of four carotenoids (violaxanthin, lutein, zeaxanthin and β-carotene) measured; P_i: inorganic phosphorus; PA:Fe: molar ratio of phytic acid to iron; FeBIO: iron bioavailability; *: significance at 0.05 level; ns: not significant.

4.3.6 Multiple regression analysis

A multiple regression was run to predict iron bioavailability from phytate, iron, lutein, violaxanthin, zeaxanthin, and β-carotene concentrations. The assumptions of linearity, independence of errors, homoscedasticity, unusual points and normality of residuals were met. These variables significantly predicted iron bioavailability, F (6, 38) = 6.26, p<.0005, $R^2 = 0.71$ (Table 4.7).

Table 4.7 Analysis of variance (ANOVA[a]) from multiple regression analysis for iron bioavailability in 4802-8 categorized sub-lines

Model	Sum of squares	DF	Mean square	F	Significance	R Square
Regression	295.6	6	49.3	5.9	0.000[b]	0.71
Residual	286.2	34	8.4			
Total	581.8	40				

[a]: Dependent variable: iron bioavailability; [b]: Predictors: (constant), phytate, iron, lutein, violaxanthin, zeaxanthin, β-carotene; DF: degree of freedom = 6 (1 dependent and 6 predictor variables were analyzed)

Lutein had significant standardized beta coefficient (0.649, p<0.0001), indicating that lutein concentration was associated with iron bioavailability (Table 4.8).

Table 4.8 Estimation of model coefficients from multiple regression analysis of iron bioavailability in 4802-8 sub-lines.

Variable	B	SE_B	ß
Intercept	16.666	8.089	
Phytate	-0.383	0.765	0.070
Violaxanthin	0.426	1.145	0.068
Lutein	1.475	0.420	0.649*
β-carotene	-6.117	1.892	-0.503
Zeaxanthin	-29.187	25.690	-0.143
Iron	-0.218	0.152	-0.181

*: $p < 0.05$; B = standardized regression coefficient; SE_B = standard error of the coefficient; ß = standardized coefficient

4.3.7 Analysis of 4803-4 categorized sub-lines

Similar to 4802-8 sub-line results, 4803-4 sub-lines, parents and CDC Bronco showed significant difference for all observed constituents except zeaxanthin concentration (Table 4.9).

Table 4.9 Mean squares of combined ANOVA, R-square, coefficient of variance (CV) and mean of different constituents in pea seeds of 4803-4 sub-lines, parents (1-150-81 and CDC Limerick) and check variety (CDC Bronco).

Source	DF	F Value	R-Square	Mean
P_i (mg g^{-1})	5	856.7*	0.97	1.2
Phytate(mg g^{-1})	5	40.0*	0.64	1.5
Iron(mg kg^{-1})	5	7.1*	0.24	48.2
Violaxanthin(mg kg^{-1})	5	4.4*	0.16	1.7
Lutein(mg kg^{-1})	5	11.9*	0.35	10.4
Zeaxanthin(mg kg^{-1})	5	1.0^{ns}	0.04	0.2
β-carotene(mg kg^{-1})	5	556.5*	0.96	0.3
TC(mg kg^{-1})	5	16.4*	0.42	12.5

P_i: inorganic phosphorus; TC: total carotenoids; *: Significance $p<0.05$; ^{ns}: Not significant; DF: degree of freedom = 5 (3 categorized sub-lines, 2 parents and CDC Bronco were analyzed)

Table 4.10 Concentration of iron, phytate and carotenoid concentration of seeds from 4803-4 categorized sub-lines, parents (1-150-81 and CDC Limerick) and check variety (CDC Bronco)

Sample	Lines	CC	PC	P_i	Phytate	Iron	Lut	Vio	Zea	β-C	TC*
				—(*mg g*$^{-1}$)—		*mg kg*$^{-1}$	——— *mg kg*$^{-1}$ ———				
4803-4 GL	22	Green	Low	1.6^{a}	1.1^{b}	49.2^{a}	11.4^{a}	2.1^{a}	0.2^{a}	0.7^{a}	14.4^{a}
4803-4 GN	20	Green	Normal	0.4^{b}	2.4^{a}	48.8^{a}	11.0^{a}	2.0^{a}	0.2^{a}	0.7^{a}	13.8^{ab}
4803-4 YL	47	Yellow	Low	1.6^{a}	1.0^{b}	48.9^{a}	10.6^{a}	1.6^{ab}	0.2^{a}	0.0^{b}	12.4^{bc}
CDC Bronco	10	Yellow	Normal	0.4^{b}	2.5^{a}	45.1^{b}	7.8^{c}	0.7^{b}	0.2^{a}	0.0^{b}	8.7^{d}
1-150-81	10	Yellow	Low	1.5^{a}	1.0^{b}	45.0^{b}	10.0^{ab}	1.8^{ab}	0.1^{a}	0.0^{b}	12.0^{bc}
CDC Limerick	10	Green	Normal	0.4^{b}	2.7^{a}	47.2^{ab}	8.9^{bc}	1.3^{ab}	0.2^{a}	0.6^{a}	10.9^{cd}
CV				8.0	36.3	5.7	13.8	53.5	19.4	22.7	14.9

CC: cotyledon color; PC: phytate category; P_i: inorganic phosphorus; Lut: lutein; Vio: violaxanthin; Zea: zeaxanthin; β-C: β-carotene; TC: total carotenoids; CV: coefficient of variation; within a column, different letters indicate significant differences at $p < 0.05$; * total carotenoid concentration was calculated as sum of four individual carotenoids.

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4.3.7.1 Phytate and inorganic phosphorus concentration

GL (1.6 mg g^{-1}) and YL (1.6 mg g^{-1}) sub-lines and 1-150-81 (1.5 mg g^{-1}) had greater mean inorganic phosphorus concentration than the normal phytate sub-lines and checks. GN sub-lines had higher phytate concentration (2.4 mg g^{-1}), among 4803-4 sub-lines and was not significantly different from CDC Bronco (2.5 mg g^{-1}) and CDC Limerick (2.7 mg g^{-1}). GL (1.1 mg g^{-1}) and YL (1.0 mg g^{-1}) had lower mean phytate concentration, which was not significantly different from 1-150-81 (1.0 mg g^{-1}) (Table 4.10).

4.3.7.2 Iron concentration

Data analysis showed that there was no statistical difference in iron concentration in 4803-4 categorized sub-lines. The low phytate parent 1-150-81 (45.0 mg kg^{-1}) and CDC Bronco (45.1 mg kg^{-1}) had lowest mean iron concentration. CDC Limerick (47.2 mg kg^{-1}) had iron concentration which was not significantly different from all samples (Table 4.10).

4.3.7.3 Carotenoids concentration

GL and GN 4803-4 sub-lines (14.4 mg $kg^{-1}$and 13.8 mg kg^{-1}, respectively) had significantly greater total carotenoids concentration than YL sub-lines (12.4 mg kg^{-1}). The categorized sub-lines from 4803-4 line showed higher lutein concentration than the parents and CDC Bronco. Higher violaxanthin concentration was found in 4803-4 categorized sub-lines and 1-150-81. All categorized 4803-4 sub-lines, parents and check variety were not statistically different from each other for zeaxanthin concentration. Green cotyledon sub-lines and parent (CDC Limerick) had higher concentration of β-carotene than yellow cotyledon sub-lines, parent (1-150-81) and check (CDC Bronco) (Table 4.10).

4.3.7.4 Iron bioavailability

Categorized GL (13.4 ng ferritin mg^{-1} of protein) and YL (12.3 ng ferritin mg^{-1} of protein) sub-lines had highest iron bioavailability followed by GN (10.2 ng ferritin mg^{-1} of protein) sub-lines and 1-150-81 (10.1 ng ferritin mg^{-1} of protein). There were significant differences for phytate and inorganic phosphorus concentrations between GN and 1-150-81, no significant difference for iron bioavailability was found (Table 4.11).

Table 4.11 Iron bioavailability (FeBIO) from 4803-4 sub-lines categorized on the basis of cotyledon color and phytate concentration, parents of 4803 cross (1-150-81 and CDC Limerick) and check variety (CDC Bronco).

Sample	number of lines	cotyledon color	phytate category	FeBIO (ng ferritin mg^{-1} of protein)
4803-4 GL	15	Green	Low	13.4[a]
4803-4 GN	15	Green	Normal	10.2[ab]
4803-4 YL	15	Yellow	Low	12.3[ab]
CDC Bronco	7	Yellow	Normal	8.3[b]
1-150-81	5	Yellow	Low	10.1[ab]
CDC Limerick	5	Green	Normal	8.3[b]
CV				30.2

CV: coefficient of variation; within a column, different letters indicate significant differences at $p < 0.05$

4.3.8 Comparison of all sub-lines with CDC Bronco

CDC Bronco is a yellow cotyledon color and normal phytate variety and progenitor of the two low phytate lines (1-2347-144 and 1-150-81); therefore, CDC Bronco was used as control in iron bioavailability experiments. Iron bioavailability was measured from three technical replicates of 15 samples in each category (Figure 4.4). Mean values showed GN sub-lines (10.2 ng ferritin mg^{-1} of cell protein) had 1.0-2.6 times higher iron bioavailability than CDC Bronco; whereas GL (13.4 ng ferritin mg^{-1} of protein) and YL (12.3 ng ferritin mg^{-1} of protein) sub-lines had up to 1.8 and 2.2 times higher iron bioavailability than CDC Bronco, respectively (Appendix 4)

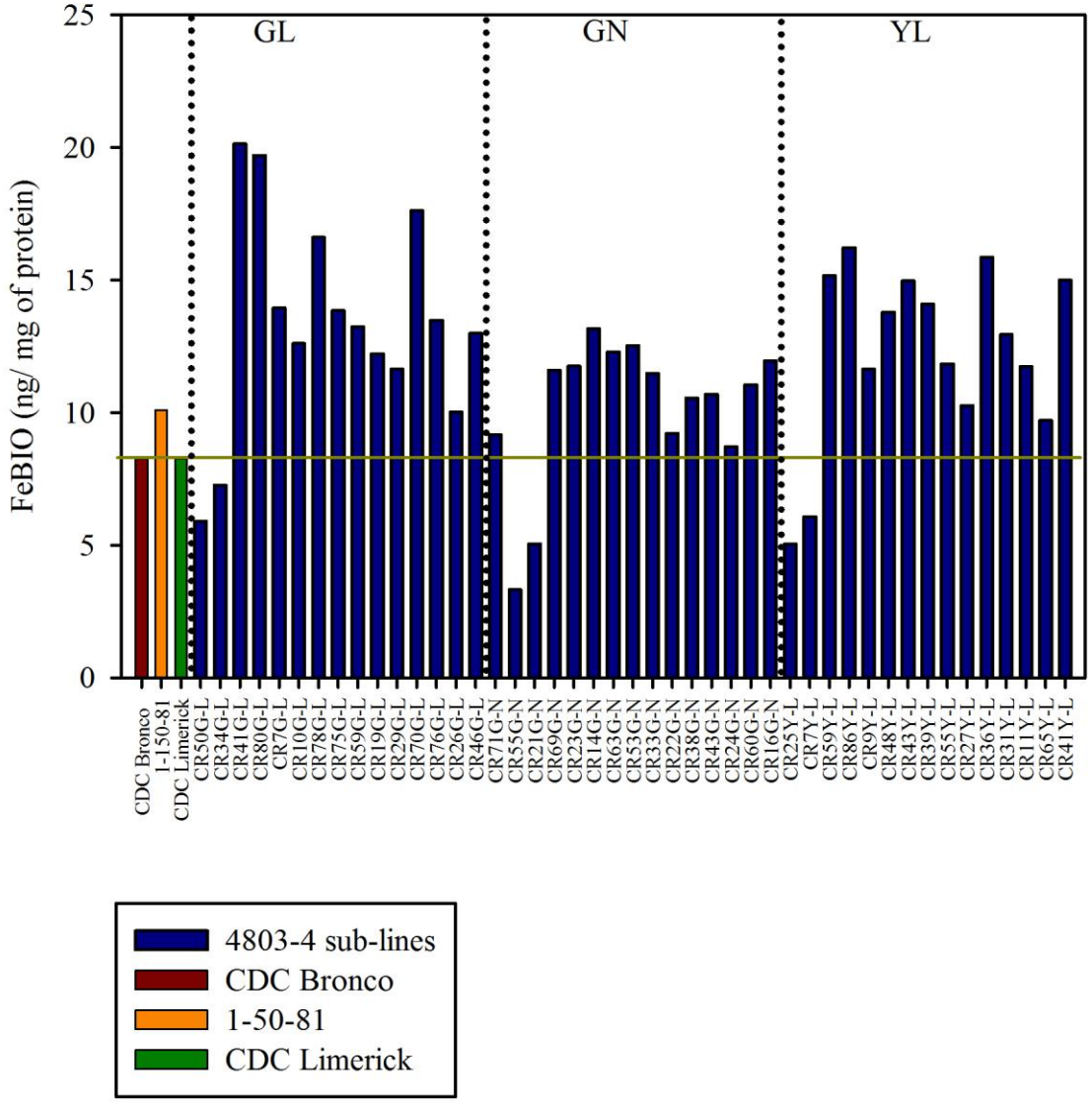

Figure 4.4 Comparison of iron bioavailability in 4803-4 sub-lines (15 samples from each) with CDC Bronco.

FeBIO: iron bioavailability; GL: Green cotyledon with low phytate ; GN : Green cotyledon with normal phytate; YL: Yellow cotyledon with low phytate; each bar represents the mean value from three technical replicates of each sample.

Similar to 4802-8 sub-lines, the two sub-lines with the highest (19.9 ± 0.31 ng ferritin mg^{-1} of protein) and the two sub-lines with the lowest (4.2 ± 1.22 ng ferritin mg^{-1} of protein) iron bioavailability were compared for phytate, iron, carotenoid concentrations and molar ratio of PA:Fe. These sub-lines differed for phytate, iron and carotenoids (lutein, violaxanthin, zeaxanthin, β-carotene) concentration and molar ratio of PA:Fe. (Table 4.12).

Table 4.12 Phytate, iron, carotenoid (lutein, violaxanthin, zeaxanthin and β-carotene) concentrations and molar ratio of phytic acid to iron of the 4803-4 sub-lines with highest and lowest iron bioavailability.

Variable	4803-4 Sub-lines mean ± SE	4803-4 Sub-lines Range	2 greatest FeBIO Sub-lines mean	2 lowest FeBIO Sub-lines mean
Phytic acid (mg g^{-1})	1.5 ± 0.13	0.0-3.8	1.2	1.6
Iron (mg kg^{-1})	48.7 ± 0.36	44.3-55.3	49.3	47.5
Molar ratio PA:Fe	9.2 ± 0.76	0.0-24.0	7.5	10.5
Lutein (mg kg^{-1})	11.0 ± 0.27	3.1-14.7	12.7	11.6
Violaxanthin (mg kg^{-1})	2.0 ± 0.92	0.4-5.0	2.0	1.2
Zeaxanthin (mg kg^{-1})	0.2 ± 0.00	0.0-0.8	0.2	0.1
β-Carotene (mg kg^{-1})	0.5 ± 0.05	0.1-0.2	0.7	0.4
Total carotenoids (mg kg^{-1})	13.6 ± 0.35	6.7-19.4	15.5	13.3
FeBIO (ng ferritin mg^{-1} of protein)	12.0 ± 0.54	3.3-20.1	19.9	4.2

SE: standard error; FeBIO: iron bioavailability

4.3.9 Correlation of several constituents with iron bioavailability in 4803-4 sub-lines

A significant positive correlation was observed between inorganic phosphorus concentration with iron bioavailability and a significant negative correlation of phytate concentration with iron bioavailability. Phytate concentration showed significant positive correlation with β-carotene concentration. Iron concentration showed negative correlation with zeaxanthin concentration. Total carotenoids and lutein concentration showed positive correlation with iron bioavailability. Phytate showed significant negative correlation with iron bioavailability (Table 4.13).

Table 4.13 Correlation matrix of violaxanthin, lutein, zeaxanthin, β-carotene, total carotenoids, inorganic phosphorus, phytate, iron, molar ratio of PA:Fe and iron bioavailability in 4803-4 sub-lines.

Variables	Violaxanthin	Lutein	Zeaxanthin	β-C	TC[a]	P_i	Phytate	Iron	PA:Fe	FeBIO
Violaxanthin	1.00	0.25^{ns}	0.04^{ns}	0.35*	0.62*	-0.15^{ns}	-0.06^{ns}	-0.13^{ns}	-0.03^{ns}	0.19^{ns}
Lutein		1.00	0.00^{ns}	0.26^{ns}	0.91*	-0.09^{ns}	-0.06^{ns}	0.00^{ns}	-0.09^{ns}	0.24^{ns}
Zeaxanthin			1.00	0.08^{ns}	0.04^{ns}	-0.08^{ns}	0.08^{ns}	-0.36*	0.04^{ns}	0.04^{ns}
β-C				1.00	0.47*	-0.44*	0.31*	-0.17^{ns}	0.32*	0.02^{ns}
TC[a]					1.00	-0.18^{ns}	-0.02^{ns}	-0.07^{ns}	-0.04^{ns}	0.26^{ns}
P_i						1.00	-0.76*	0.12^{ns}	-0.71*	0.36*
Phytate							1.00	0.07^{ns}	0.89*	-0.37*
Iron								1.00	-0.03^{ns}	0.06^{ns}
PA:Fe									1.00	-0.38*
FeBIO										1.00

β-C: β-carotene; TC: total carotenoids; [a]: sum of four carotenoids (violaxanthin, lutein, zeaxanthin and β-carotene) measured; P_i: inorganic phosphorus; PA:Fe: molar ratio of phytic acid to iron; FeBIO: iron bioavailability; *: significance at 0.05 level; ns: not significant.

4.3.10 Multiple regression analysis

A multiple regression was run to predict iron bioavailability from phytate, iron, lutein, violaxanthin, zeaxanthin and β-carotene concentration. The assumptions of linearity, independence of errors, homoscedasticity, unusual points and normality of residuals were met. These variables did not significantly predicted iron bioavailability, F (6, 38) = 1.66, p>.05, R^2 = 0.21. Phytate had the highest standardized beta coefficient (-0.369, p=0.02), indicating that phytate concentration had an association with iron bioavailability (Table 4.14 and 4.15).

Table 4.14 Analysis of variance (ANOVA[a]) from multiple regression analysis for iron bioavailability in 4803-4 categorized sub-lines

Model	Sum of squares	DF	Mean square	F	Significance	R Square
Regression	118.9	6	19.8	1.66	0.16[b]	0.21
Residual	452.5	38	11.9			
Total	571.4	44				

[a]: Dependent variable: iron bioavailability; [b]: Predictors: (constant), phytate, iron, lutein, violaxanthin, zeaxanthin, β-carotene; DF: degree of freedom = 6 (1 dependent and 6 predictor variables were analyzed)

Table 4.15 Estimation of model coefficients from multiple regression analysis of iron bioavailability in 4803-4 sub-lines.

Variable	B	SE_B	ß
Intercept	-2.463	13.523	
Phytate	-1.627	0.689	-0.369*
Violaxanthin	0.517	0.650	0.126
Lutein	0.322	0.308	0.161
β-carotene	0.643	1.912	0.057
Zeaxanthin	12.488	21.813	0.088
Iron	0.206	0.239	0.136

*: $p < 0.05$; B = standardized regression coefficient; SE_B = standard error of the coefficient; ß = standardized coefficient

Carotenoid concentrations found in green and yellow cotyledon sub-lines (both 4802-8 and 4803-4) were compared with the concentrations observed in different cultivars by Ashokkumar et al (2014) (Figure 4.5). Lutein and β-carotene concentrations in green cotyledon sub-lines (both 4802-8 and 4803-4) were lower than in the green cultivars reported by Ashokkumar et al (2014). There was no difference in zeaxanthin concentration in sub-lines and cultivars, while violaxanthin concentration was higher in all sub-lines compared to cultivars reported by Ashokkumar et al (2014). Total carotenoid concentration in all sub-lines was lower than in green cotyledon cultivars, but greater than in yellow cotyledon cultivars (Figure 4.5). In Ashokkumar et al (2014) four green pea cultivars (CDC Tetris, CDC Patrick, CDC Striker and Cooper) were evaluated, whereas in the current study, the sub-lines were derived from crosses with two other green pea parents (CDC Raezer and CDC Limerick) and it is plausible that substantial variation exists in green pea germplasm for carotenoid concentration.

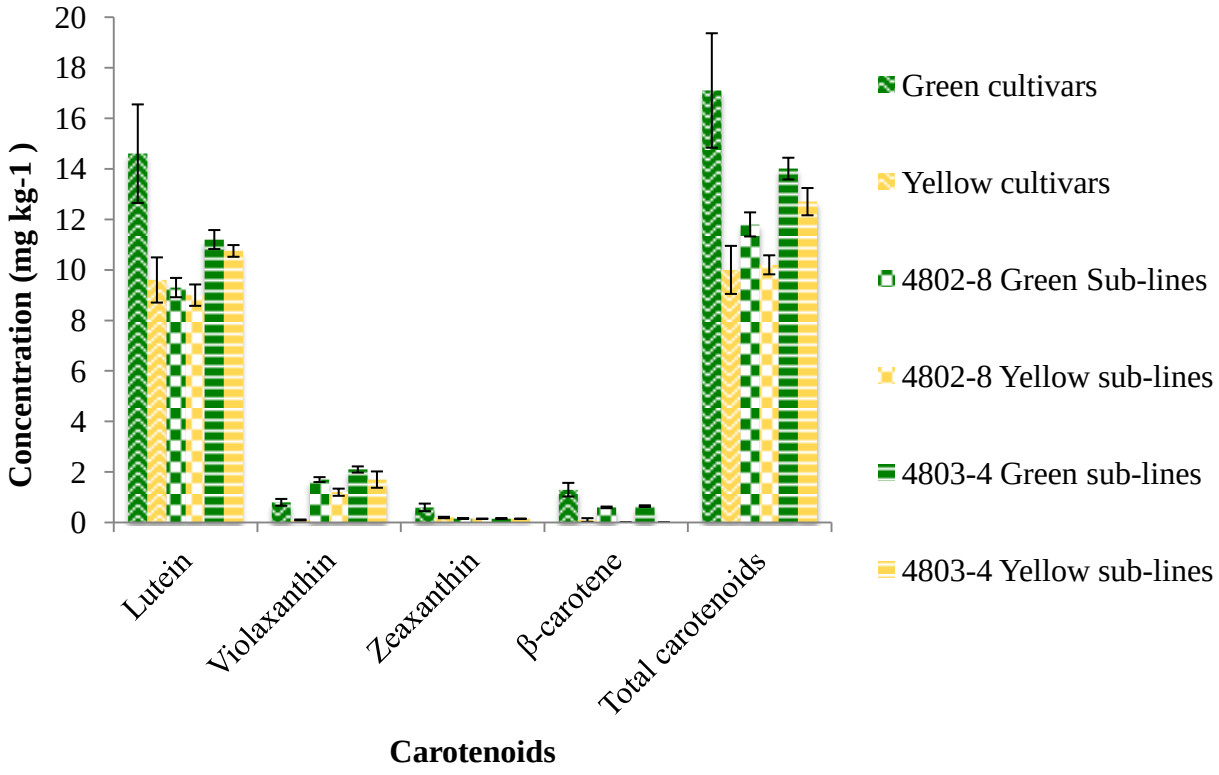

Figure 4.5 Comparison of lutein, violaxanthin, zeaxanthin, β-carotene and total carotenoid (TC) concentrations between cultivars (green and yellow cotyledon) used by Ashokkumar et al (2014) and sub-lines (4802-8 and 4803-4)

The pattern of change in iron bioavailability with change in molar ratio of PA:Fe was not significant. Although the molar ratio (PA:Fe) ranged from 0.02:1 to 24:1, a non-significant regression (0.03) was observed in 4802-8 sub-lines. Whereas, in the 4803-4 sub-lines, a significant, but weak, regression (0.14) of molar ratio (PA:Fe) with iron bioavailability was observed (Figure 4.6).

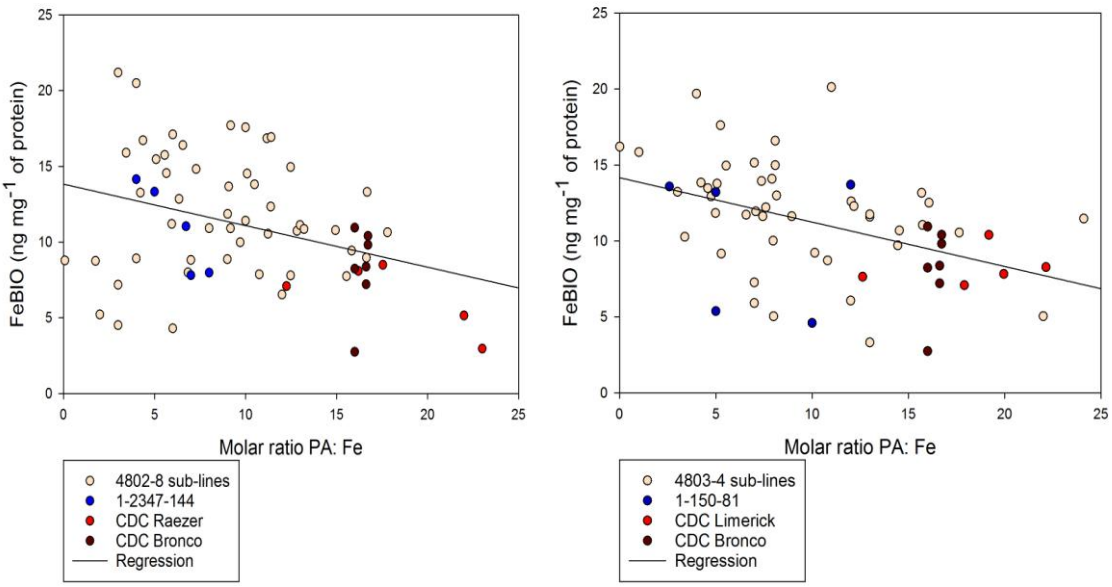

Figure 4.6 Changes in FeBIO (iron bioavailability) with the change on molar ratio of phytic acid (PA) to iron (Fe) in 4802-8 and 4803-4 sub-lines

4.4 Discussion

This study was conducted to evaluate the potential additive effect of phytate and carotenoid concentrations on iron bioavailability in pea seeds, as their different concentrations were observed to enhance iron absorption individually in previously reported studies. In this study, pea lines contrasting for phytate concentration (low or normal) as well as cotyledon color (green or yellow) were selected. Phytate concentration in all sub-lines ranged from trace amounts to 3.8 mg g^{-1}, which is comparable to the values previously reported by Liu et al., 2014 (ranging from 1.2 to 3.0 mg g^{-1}) and Ravindran et al., 1994 (ranging from 2.2 to 12.2 mg g^{-1}). The low phytate sub-lines had 43 to 58 % lower phytate concentration than the normal phytate sub-lines. This was also observed in other *lpa* mutants of common bean (*Phaseolus vulgaris* L.), maize (*Zea mays* L.), rice (*Oryza sativa* L.), barley (*Hordeum vulgare* L.), and field pea (*Pisum sativum* L.), which showed up to 90 %, 66 %, 64 %, 45 % and 50 % reduction in phytate concentration, respectively (Campion et al., 2009; Raboy et al., 2000; Shi et al., 2003; Shi et al., 2005; Larson et al., 2000; Larson et al., 1998; Veum et al., 2007; Warkentin et al., 2012). Loss of yellow cotyledon with normal phytate category could be due to segregation or error in classification (Table 4.1). The low phytate concentration was associated with increased inorganic phosphorus concentration up to 2 to 3 times in the sub-lines. While there was a negative correlation between phytate and inorganic phosphorus concentration, the total phosphorus concentration was similar among all categorized sub-lines (Table 4.1; Figure 4.1 and 4.2). Iron concentration ranged from 42 to 59 mg kg^{-1} and no significant difference was observed among categorized sub-lines (Table 4.3 and 4.10). In the current study, iron bioavailability in categorized low phytate sub-lines was up to 2.5 times greater as compared to CDC Bronco (normal phytate cultivar). The magnitude of increase in iron bioavailability was greater than in previous reports, where low phytate pea mutant lines (1-150-81 and 1-2347-144) showed an increase of 1.5 to 2 times in iron

bioavailability compared to CDC Bronco (Liu et al., 2014). About 60 % reduction in phytate concentration in maize *lpa*1-1 resulted in 1.5 fold higher iron bioavailability (~30 ng/ mg of protein) compared to normal phytate parent (20 ng/ mg of protein) (Aluru et al., 2011). Similarly, the removal of phytates in soy protein isolate increased iron absorption by 4 to 5 times (Hurrell et al., 1992). In lentil study, dehulled seeds showed higher iron bioavailability than whole seeds. Iron concentration in dehulled seeds was 46-69 mg kg^{-1}, while iron bioavailability ranged from 5-12 ng/ mg of protein (Della Valle et al., 2013). In contrast, in the present study, peas had comparatively lower iron concentration ranging from 42-59 mg kg^{-1} but showed higher iron bioavailability ranging from 5-22 ng/ mg of protein than lentils. In another study on lentil genotypes obtained from Saskatchewan, large range for iron concentration (43-92 mg kg^{-1}) and its bioavailability (1.7-44.3 ng/ mg of protein) was observed. In common beans, lectin free and low phytate white colored lines/ sub-lines had mean iron concentration of 79 mg kg^{-1} with iron bioavailability of 28 ng/ mg of protein (Campion et al., 2013).

The molar ratio of PA:Fe is an important predictor of iron absorption from digested food. PA:Fe molar ratio greater than 10:1 inhibited micronutrient bioavailability (Glahn et al., 2002; Turnlund et al., 1984). Glahn et al (2002) reported when molar ratio PA:Fe ranged from 3:1 to 10:1, limited inhibition of iron absorption was observed. However, this trend was not observed in the present study. Although PA:Fe molar ratio went up to 24:1, inhibition of iron absorption was not observed (Figure 4.6). In previous research, normal phytate cultivars (CDC Bronco, CDC Golden and CDC Meadow) had higher molar ratio (ranged from 18:1 to 25:1) and they showed up to 1.9 times lower iron bioavailability than 1-2347-144 and 1-150-81, which had lower molar ratio (10:1 to 14:1) (Liu et al., 2014). Sub-lines of 4802-8 and 4803-4 with low molar ratio

(0.02:1 to 2:1) ranged from low (5.2 ng ferritin mg^{-1} of protein) to high (16.2 ng ferritin mg^{-1} of protein) in iron bioavailability (Figure 4.6).

Ashokkumar et al (2014) observed green cotyledon (18.4 mg kg^{-1}) pea cultivars had approximately twice the total carotenoid concentration compared to yellow cotyledon (9.7 mg kg^{-1}) pea cultivars (Figure 4.5). This trend was not observed in the present study, where green and yellow cotyledon sub-lines had similar concentration of total carotenoids (Table 4.3 and 4.10). Lutein was the dominant carotenoid compound among all carotenoids present in pea seed in this study and in that of Ashokkumar et al (2014). Lutein concentration in 4802-8 and 4803-4 sub-lines ranged from 3.9 to 13.2 mg kg^{-1}, and 3.1 to 14.7 mg kg^{-1}, respectively. The concentrations were comparable to the values reported previously, where lutein concentration varied from 3.5-13.9 mg kg^{-1} (Holosavá et al., 2009; Ashokkumar et al., 2014; Marles et al., 2013). Violaxanthin and zeaxanthin concentrations were also comparable to values reported previously. The HPLC chromatogram of carotenoid profile showed β-carotene at 22.0 min and was observed only in green cotyledon sub-lines (Figure 3.2 a). Lutein, violaxanthin, zeaxanthin and total carotenoid concentration were not significantly different in green and yellow cotyledons of 4802-8 and 4803-4 sub-lines, but higher β-carotene concentration was found in green cotyledon pea sub-lines (Table 4.3 and 4.10). Previous studies such as Holasová et al (2009) and Ashokkumar et al (2014) reported 10 times higher concentration of β-carotene in green cotyledon compared to yellow or orange cotyledon pea cultivars. However, β-carotene concentration in green sub-lines (Table 4.3 and 4.10) was lower than concentrations reported by Ashokkumar et al (2014) (Figure 4.5) and Holasová et al (2009). This may be because diverse cultivars were used by Ashokkumar et al. (2014), while in the current study, sub-lines of two specific breeding lines were used. While different cultivars may have different concentration of

total carotenoids, the sub-lines from a single line are expected to have more uniform carotenoid concentration.

In the current study, lutein concentration was positively correlated with iron bioavailability in both sub-lines (4802-8 and 4803-4) (Table 4.6 and 4.13), and this trend was also observed by García-Casal et al (2006). β-carotene concentration had no significant correlation with iron bioavailability in both sub-lines (Table 4.6 and 4.13). This is in contrast with results from García-Casal et al (2000) and Layrisse et al (1997) which showed β-carotene overcame the inhibitory effect of phytate and its supplementation in corn and wheat flours doubled iron absorption by Caco-2 cells. In 4802-8 sub-lines, iron bioavailability had negative and positive correlation with phytate and lutein concentrations, respectively (Table 4.6). The effect of phytate was more substantial than the effect of lutein in 4803-4 sub-lines. It is possible that carotenoids have positive effects on iron bioavailability, but substantial contrast for carotenoid concentration was not observed among the sub-lines used in the current study.

CHAPTER 5

GENERAL DISCUSSION

Approximately one third of the world's population suffers from micronutrient malnutrition (Food and Agriculture Organization, 2012). Iron, zinc and vitamin A are three critical nutrients, which are the most limiting in human diets (World Health Organization, 2002; Bouis, 2003). Iron deficiency is a major health concern and is highly prevalent among infants, children and women worldwide. Iron deficiency anemia results in child and maternal mortality, decrease mental development and work capacity and the body becomes susceptible to infectious diseases. The main causes for iron deficiency are reliance on plant based diets, blood loss during menstrual cycle, pregnancy and parasitic infections. Non-heme dietary iron from cereal and legume based diets has low bioavailability and is considered as a major factor for iron deficiency anemia. The most common and costly strategies used for reducing iron deficiency are supplementation (tablets, capsules, syrups) and food fortification (with specific micronutrients). However, production of crops with high iron density through plant breeding is a low-cost, sustainable approach and provides feasibility of reaching malnourished populations. QTL associated with iron concentration could be useful in marker assisted breeding to develop iron dense crop varieties. Field pea (*Pisum sativum* L.) is a nutritious legume crop grown for human and animal consumption world-wide. In PR-07 pea recombinant inbred line population, three QTL associated with iron concentration were identified on LG3, LG4 and LG7 with GoldenGate array linkage map, and finely mapped on a GBS linkage map. A single locus for cotyledon color was mapped on LG1 using the GBS linkage map. The key results from this study are that iron concentration is a quantitatively inherited trait and can be mapped on the PR-07 linkage map. The QTL associated with iron concentration and cotyledon color were not linked to each other.

Therefore, the first hypothesis, QTL associated with iron concentration can be found in pea recombinant inbred line population segregating for iron concentration, is accepted.

Iron bioavailability in field pea seeds depends on two factors: the iron concentration and its interaction with antinutrients and enhancers, which affect its absorption in the gut. Phytate is an antinutrient, which chelates iron and reduces its bioavailability in monogastric animals which lack phytase enzyme in their digestive tract (Reddy et al., 1982). Carotenoids (lutein, lycopene, zeaxanthin, and β-carotene) enhanced iron bioavailability from cereal based diets (García-Casal et al., 2006; García-Casal, 1998).

Increasing iron concentration with food fortification increased iron bioavailability (Layrisse et al., 1996; Hurrell, 1997). Does higher iron concentration in food itself increase the bioavailable amount of iron? To answer this question, the patterns of iron bioavailability with the change in iron concentration were studied. The selected lines were segregating for iron concentration and cotyledon color. Although iron bioavailability did not differ significantly in high vs. low iron categories, a significant correlation was observed between iron concentration and its bioavailability in PR-07. So the second hypothesis, pea seeds with greater iron concentration may have greater iron bioavailability, is accepted.

The second study was conducted to evaluate the potential additive effect of phytate and carotenoid concentration on iron bioavailability. No significant correlation was detected between individual carotenoids with iron bioavailability, except for a significant positive correlation between lutein and iron bioavailability in the 4802-8 sub-lines. So the third hypothesis, pea seeds with greater carotenoid concentration may have greater iron bioavailability, is accepted.

In both 4802-8 and 4803-4 sub-lines, categorized low phytate lines had iron bioavailability up to 2.5 times greater than CDC Bronco (normal phytate pea cultivar), while phytate and lutein

concentration showed significant negative and positive correlation with iron bioavailability, respectively. Significant correlations were not detected between other carotenoids or total carotenoid concentration with iron bioavailability. This observation is not surprising because substantial contrast for carotenoid concentration was not observed among sub-lines used in the present study. Moreover, the coefficient of variation for iron bioavailability was relatively high, ranging from 27.6 to 30.2 (Table 4.4 and 4.11), indicating the inherent variability within the Caco-2 assay. This variability could have prevented detection of minor differences in iron bioavailability among sub-lines. Therefore, the fourth hypothesis, low phytate concentration and high carotenoid concentration in pea seeds may have additive benefits for iron bioavailability, is partially accepted. These results suggest that selecting low phytate pea lines with greater iron and carotenoid concentration may result in increased iron bioavailability for humans and monogastric animals. In common beans, genetic selection of varieties for higher iron concentration had increased iron bioavailability (Welch et al., 2000). This research also suggests that factors (antinutrients or enhancers) other than concentration of iron, phytate and carotenoids may affect iron bioavailability.

Suggested future research related to this project include the following:

1. Develop and implement KASP assays for iron concentration and phytate concentration in pea RIL populations segregating for these traits and in a pea association mapping panel.
2. Chicken feeding trail with most promising sub-lines (i.e. with the highest iron bioavailability) from 4802-8 and 4803-4 to confirm the findings from Caco-2 cell culture assays.
3. Estimation of iron bioavailability in pea varieties which contrast substantially for carotenoid concentration.

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APPENDICES

Appendix 1. Mean of Days to flower (DTF), E (Emergence percent), PH (plant height), Lodging, MB1(first Mycosphaerella blight score), MB2 (second Mycosphaerella blight), Days to maturity (DTM), grain yield and thousand seed weight of 4802-8 categorized sub-lines grown at Sutherland, Saskatoon in 2014

4802-8	DTF (days)	E (%)	PH (cm)	Lodging (1-9 score)	MB1 (0-9 score)	MB2 (0-9 score)	DTM (days)	Grain Yield (g)	1000 Seed Weight
Green Low	56[a]	69[a]	68[a]	3.9[a]	4.1[a]	4.8[a]	96[ab]	430[abc]	226[bc]
Green Normal	55[a]	63[a]	64[a]	3.5[a]	3.5[a]	4.6[a]	95[c]	391[c]	245[a]
Yellow Low	55[a]	65[a]	66[a]	4.0[a]	3.8[a]	4.6[a]	95[bc]	426[bc]	246[a]
CDC Bronco	56[a]	73[a]	67[a]	3.6[a]	3.6[a]	4.4[a]	96[abc]	526[a]	242[ab]
1-2347-144	56[a]	61[a]	70[a]	3.3[a]	3.6[a]	4.5[a]	98[a]	501[ab]	209[c]
CDC Raezer	52[b]	70[a]	63[a]	4.4[a]	4.4[a]	5.2[a]	94[c]	367[c]	230[abc]
CV	4.1	15.3	11.8	28.2	22.6	16.2	1.9	21.1	7.8

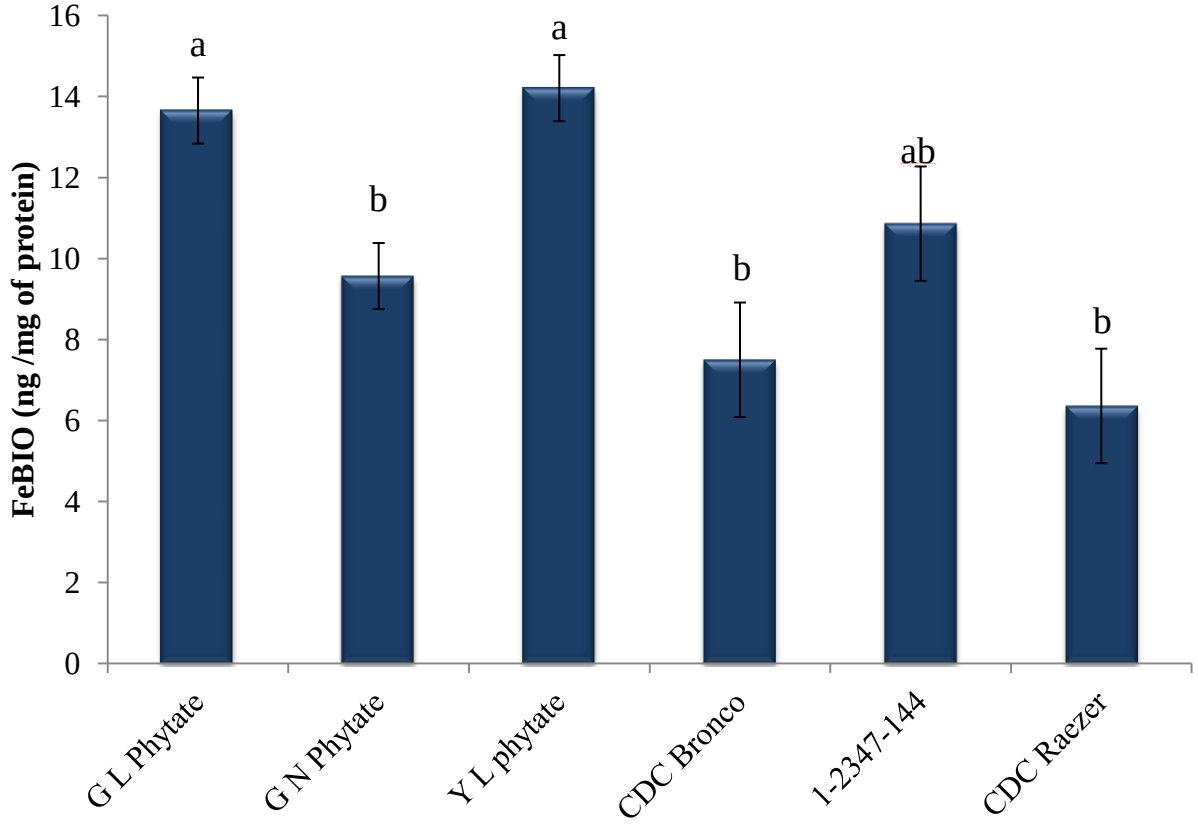
CV: coefficient of variation, Lodging score 1= Main stems strictly upright, 9 = All main stems flat, MB (0 = free of disease on leaves/stems), MB (9 = severe infection)

Appendix 2. Mean of Days to flower (DTF), E (Emergence percent), PH (plant height), Lodging, MB1(first Mycosphaerella blight score), MB2 (second Mycosphaerella blight), Days to maturity (DTM), grain yield and thousand seed weight of 4803-4 sub-lines grown at Sutherland, Saskatoon in 2014

4803-4	DTF (days)	E (%)	PH (cm)	Lodging (1-9 score)	MB1 (0-9 score)	MB2 (0-9 score)	DTM (days)	Grain Yield (g)	1000 Seed Weight
Green Low	56^{ab}	74^{a}	74^{a}	3.5^{a}	3.7^{ab}	4.8^{ab}	96^{a}	575^{a}	235^{ab}
Green Normal	56^{a}	74^{a}	75^{a}	3.2^{a}	4.2^{a}	4.9^{a}	96^{a}	580^{a}	223^{bc}
Yellow Low	57^{a}	67^{ab}	72^{ab}	3.1^{a}	3.3^{b}	4.4^{ab}	96^{a}	537^{ab}	236^{a}
CDC Bronco	56^{ab}	73^{ab}	67^{b}	3.6^{a}	3.4^{ab}	4.3^{ab}	96^{a}	526^{ab}	242^{a}
1-150-81	56^{ab}	64^{ab}	70^{ab}	3.1^{a}	3.3^{ab}	4.2^{ab}	97^{a}	496^{ab}	217^{c}
CDC Limerick	54^{b}	61^{b}	74^{ab}	2.7^{a}	2.9^{b}	4.0^{b}	97^{a}	457^{b}	215^{c}
CV	3.9	14.7	7.6	29.8	28.0	17.6	1.5	16.9	6.0

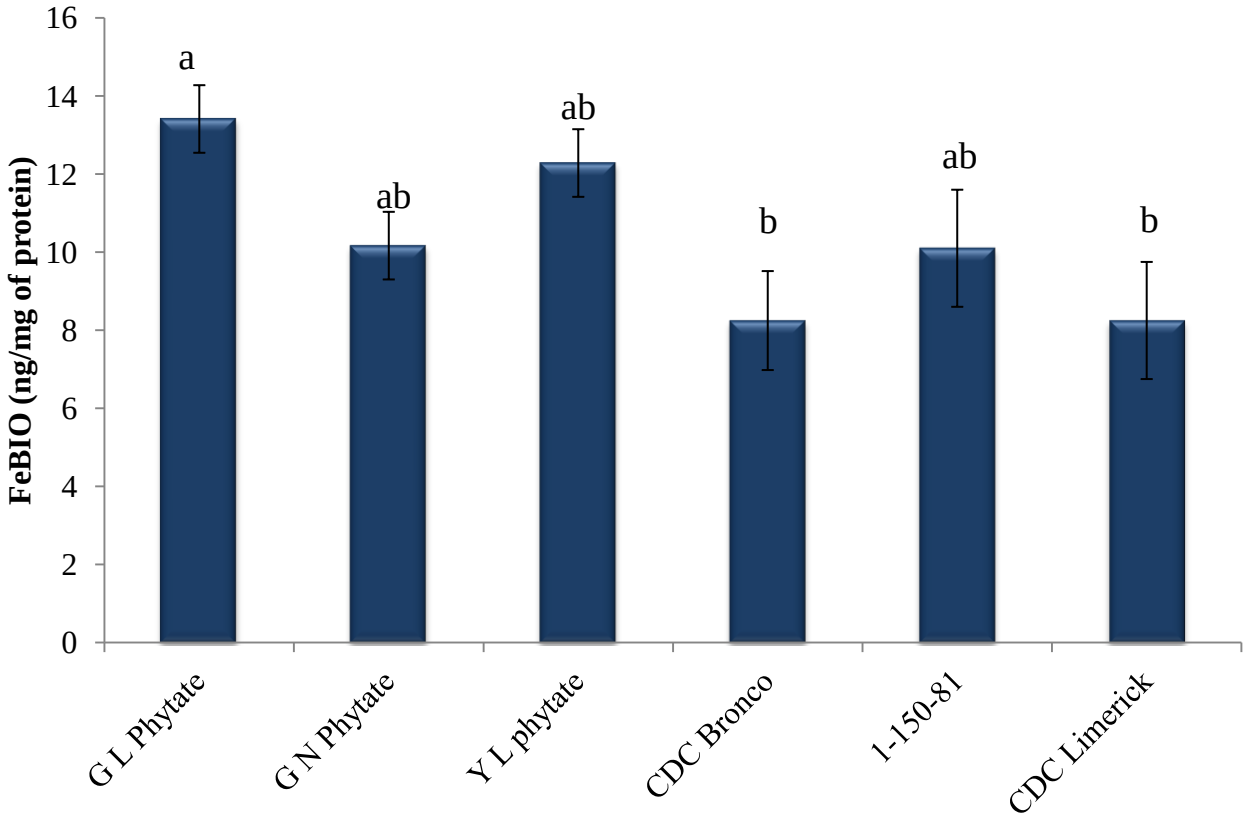
CV: coefficient of variation, Lodging score 1= Main stems strictly upright, 9 = All main stems flat, MB (0 = free of disease on leaves/stems), MB (9 = severe infection)

Appendix 3. Comparison of iron bioavailability (FeBIO) between categorized 4802-8 sub-lines (mean of 15 per category), parents of 4802 cross (1-2347-144 and CDC Raezer) and check variety (CDC Bronco)



GL: Green cotyledon with low phytate ; GN : Green cotyledon with normal phytate; YL: Yellow cotyledon with low phytate; Significance at P<0.05

Appendix 4. Comparison of iron bioavailability (FeBIO) between categorized 4803-4 sub-lines (mean of 15 per category), parents of 4803 cross (1-150-81 and CDC Limerick) and check variety (CDC Bronco)



GL: Green cotyledon with low phytate ; GN : Green cotyledon with normal phytate; YL: Yellow cotyledon with low phytate; Significance at P<0.05